



King's Research Portal

DOI:

[10.1016/j.ebiom.2019.03.080](https://doi.org/10.1016/j.ebiom.2019.03.080)

Document Version

Peer reviewed version

[Link to publication record in King's Research Portal](#)

Citation for published version (APA):

Pellizzari, G., Hoskin, C. M., Crescioli, S., Mele, S., Gotovina, J., Chiaruttini, G., Bianchini, R., Ilieva, K. M., Bax, H. J., Papa, S. E., Lacy, K. E., Jensen-Jarolim, E., Tsoka, S., Josephs, D. H., Spicer, J. F., & Karagiannis, S. N. (2019). IgE re-programs alternatively-activated human macrophages towards pro-inflammatory anti-tumoural states. *EBioMedicine*, 43, 67-81. [EBIOM2073]. <https://doi.org/10.1016/j.ebiom.2019.03.080>

Citing this paper

Please note that where the full-text provided on King's Research Portal is the Author Accepted Manuscript or Post-Print version this may differ from the final Published version. If citing, it is advised that you check and use the publisher's definitive version for pagination, volume/issue, and date of publication details. And where the final published version is provided on the Research Portal, if citing you are again advised to check the publisher's website for any subsequent corrections.

General rights

Copyright and moral rights for the publications made accessible in the Research Portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognize and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the Research Portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the Research Portal

Take down policy

If you believe that this document breaches copyright please contact librarypure@kcl.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.

IgE re-programs alternatively-activated human macrophages towards pro-inflammatory anti-tumoural states

Giulia Pellizzari¹, Coran Hoskin², Silvia Crescioli¹, Silvia Mele¹, Jelena Gotovina^{3,4}, Giulia Chiaruttini¹, Rodolfo Bianchini^{3,4}, Kristina Ilieva^{1,6}, Heather J Bax^{1,5}, Sophie Papa^{5,7}, Katie E Lacy¹, Erika Jensen-Jarolim^{3,4}, Sophia Tsoka², Debra H Josephs^{5,7}, James F Spicer⁵, Sophia N Karagiannis¹

¹ St. John's Institute of Dermatology, School of Basic and Medical Biosciences, King's College London, Guy's Hospital, London, SE1 9RT, United Kingdom;

² Department of Informatics, Faculty of Natural and Mathematical Sciences, King's College London, Bush House, London, WC2B 4BG, United Kingdom;

³ Institute of Pathophysiology and Allergy Research, Center for Pathophysiology, Infectiology and Immunology, Medical University Vienna, Austria;

⁴ Department of Comparative Medicine, The Interuniversity Messerli Research Institute of the University of Veterinary Medicine Vienna, Medical University Vienna and University Vienna, Vienna, Austria;

⁵ School of Cancer & Pharmaceutical Sciences, King's College London, Bermondsey Wing, Guy's Hospital, London SE1 9RT, United Kingdom;

⁶ Breast Cancer Now Research Unit, School of Cancer & Pharmaceutical Sciences, King's College London, Guy's Cancer Centre, London, United Kingdom;

⁷ Guy's and St Thomas' NHS Trust, Department of Medical Oncology, London, United Kingdom

Corresponding Author:

Sophia N. Karagiannis, PhD, St. John's Institute of Dermatology, School of Basic and Medical Biosciences, King's College London, Guy's Hospital, Tower Wing, 9th Floor, London, SE1 9RT, United Kingdom

E-mail: sophia.karagiannis@kcl.ac.uk

Key Words: Cancer Immunotherapy, IgE, Macrophages, Alternatively-activated macrophages, AllergoOncology

Abstract (250 words)

Background: Antibody Fc-driven engagement of macrophages is critical for evoking cellular activation and effector functions and influencing tumour-associated macrophage (TAM) recruitment. We previously reported that IgE class antibodies promote restriction of cancer growth in rodent models associated with significant TAM infiltration. However, the human macrophage-associated IgE-Fc Receptor (FcεR) axis remains unexplored. We investigated the effects of anti-tumour IgE stimulation on human macrophage activation.

Methods: Human blood monocyte-differentiated quiescent (M0), classically-(M1) and alternatively-(M2) activated macrophages were crosslinked with IgE and polyclonal antibodies to mimic immune complex formation. We examined surface marker expression, cytokine secretion, protein kinase phosphorylation and gene expression in IgE-stimulated macrophages and IgE antibody-dependent macrophage-mediated cytotoxicity (ADCC) against tumour cells.

Findings: A proportion (40%) of M2 and (<20%) M0 and M1 macrophages expressed the high-affinity IgE receptor FcεRI. IgE crosslinking triggered upregulation of co-stimulatory CD80, increased TNFα, IFNγ, IL-1β, IL-12, IL-10, IL-13, CXCL9, CXCL11 and RANTES secretion by M0 and M2 and additionally enhanced MCP-1 by M2 macrophages. IgE-stimulated M1 macrophages retained secretion of pro-inflammatory cytokines. IgE crosslinking enhanced the FcεRI-dependent signalling pathway, including phosphorylation of the Lyn kinase, ERK1/2 and p38 in M2 macrophages and upregulated *Lyn* gene expression by M1 and M2 macrophages. Anti-tumour IgE engendered ADCC of cancer cells by all macrophage subsets.

Interpretation: IgE can engage and re-educate alternatively-activated macrophages towards pro-inflammatory phenotypes and prime all subsets to mediate anti-tumour functions. This points to IgE-mediated cascades with potential to activate immune stroma and may be significant in the clinical development of strategies targeting tumour-resident macrophages.

Introduction

Tumour-associated macrophages (TAMs) form a significant proportion of the immune cell infiltrate and are thought to play important roles in defining the pro- or anti-tumour status of the tumour microenvironment (TME). The mechanisms by which macrophages can influence tumorigenesis, though, has been controversial: these cells represent a multi-faceted immune compartment and their contributions in shaping a pro- or anti-tumour environment heavily depend on their polarisation status ⁽¹⁾. While the conventional binary model of macrophage polarisation of classically-activated (M1) and alternatively-activated M2 subsets may be imprecise, generally tumour-associated macrophage infiltrates are thought to favour an alternatively-activated M2-like phenotype. TAMs, featuring M2 characteristics, have been demonstrated to permit or promote tumour growth and their presence in the tumour tissue has been associated with poor prognosis in some solid tumours of various origins, such as melanoma, head and neck, lung, colorectal and breast cancers ⁽²⁾. For these reasons, TAMs are now considered a potential target for cancer immunotherapy ⁽³⁻⁶⁾.

Several anti-cancer approaches targeting macrophages have been explored aiming to either inhibit their tumour-supporting functions or to promote their anti-tumour effects. More specifically, macrophage-centred anti-cancer strategies can include macrophage depletion, modification of macrophage recruitment and macrophage re-polarization or re-programming. Macrophage reprogramming involves the suppression of alternatively-activated (M2) macrophages or, possibly, the stimulation of an M1-biased phenotype in the TME ⁽⁷⁾. The development of a monoclonal antibody targeting the pattern recognition scavenger MARCO ⁽⁸⁾ is the first antibody therapeutic approach designed to re-programme TAMs.

The demonstration that re-polarisation of macrophages can be achieved through monoclonal antibody treatment opened the door to the development of new therapeutics targeting macrophage surface proteins (specifically those involved in maintaining an M2-like phenotype). We previously demonstrated that treatment with MOv18 IgE, a monoclonal human/chimeric antibody engineered with Fc regions of the IgE class and specific for a tumour-associated antigen (TAA) (Folate Receptor alpha, FR α), triggered an immune-activatory TNF α /MCP-1 axis in tumours, which resulted in recruitment of macrophages into tumour lesions and was associated with significantly-reduced tumour growth ⁽⁹⁾, in a syngeneic immunocompetent lung metastases rat model. These findings suggested that IgE antibodies may activate and recruit macrophages towards tumours and thus may represent a treatment approach capable of engaging these cells against cancer ^(3,10). However, while activation of mast cells and subsequent release of pro-inflammatory mediators have been largely described ^(11,12), very little is known regarding IgE-Fc ϵ Receptor interactions and their downstream effects on macrophage activation and functions, especially with regards to distinct macrophage subpopulations ⁽¹³⁾.

In our study we investigated how a monoclonal antibody (SF-25 IgE) targeting a TAA, can engage and activate human macrophages of different activation states. We investigated human macrophage expression of IgE receptors, the potential effects of IgE crosslinking on surface marker expression, soluble mediator release, phosphorylation and gene expression of kinases known to be associated with Fc ϵ R signalling. Furthermore, we investigated whether macrophages of different polarisation states can trigger effector functions against cancer cells by anti-tumour IgE, with the aim of ascertaining the potential of antibodies of the IgE class to promote macrophage-mediated anti-tumour activity.

Materials and Methods

Isolation of human peripheral blood mononuclear cells (PBMC)

K₂EDTA-spray coated collection tubes were used to collect peripheral venous blood (50 ml) from healthy donors. To isolate PBMCs, equal volumes of blood and 2% FCS/2 mM EDTA were gently mixed to a final volume of 30 ml and gently pipetted on top of 15 ml of Ficoll-PaqueTM PLUS density gradient in a 50 ml conical tube. The tube was then centrifuged at 1200 x g with slow acceleration and no brake at room temperature (RT) for 20 minutes. The plasma interface was collected and washed with PBS at 600 x g at 4°C for 10 minutes. The erythrocytes present in the sample were lysed with RBC lysis buffer for 5 minutes at RT, followed by washing with PBS + 2% FCS/2 mM EDTA. Human samples were collected with informed written consent, in accordance with the Helsinki Declaration. Study design was approved by the Guy's Research Ethics Committee, Guy's and St. Thomas' NHS Foundation Trust. Peripheral blood was also obtained through the UK National Health System (NHS) Blood and Transplant system from anonymous donor leukocyte cones.

Isolation of human monocytes from peripheral blood

Isolated PBMCs were passed through a 40 µm cell strainer to remove cell clumps. Next, PBMCs were transferred in a new vial and washed with PBS at 600 x g for 5 minutes. After discarding the supernatant, cells were resuspended in 400 µl of MACS buffer (PBS + 0.5% FCS/2 mM EDTA). Human monocytes were isolated by negative selection, following the Pan Monocyte Isolation Kit protocol (Miltenyi Biotec). The purity of monocytes was evaluated by flow cytometry staining with a BV786-conjugated monoclonal antibody anti-CD14 and BV711-conjugated anti-CD16. Acquisitions were performed using a BD FACS CantoTM II at the Biomedical Research Centre Flow Cytometry Core, King's College London.

Differentiation of human macrophages *ex vivo* from peripheral blood monocytes

Isolated monocytes were resuspended in RPMI 1640 2% FBS to promote adhesion and cells were seeded in 6-well plates at a density of 1-1.5 x 10⁶ cells/ml; each well was loaded with 2 ml of cell suspension and plates were placed in a tissue culture incubator. After 2 hours of incubation, medium was carefully removed. Each well was washed with 1 ml of warm sterile PBS and replenished with 2 ml of RPMI 1640 supplemented with 10% FCS and penicillin/streptomycin with the addition of 20 ng/ml of M-CSF (Monocyte Colony Stimulating Factor) to allow monocyte to grow *ex vivo*. Thereafter, every 3 days, half of the volume in each well fresh were replaced with media containing 25 ng/ml GM-CSF (for M1) or 40 ng/ml M-CSF (for M2). On Day 7, 20 ng/ml of Interferon-Gamma (IFN γ) and 100 ng/ml of Lipopolysaccharide (LPS) were added to GM-CSF cells for 72 hours. M-CSF cells were instead added with 20 ng/ml of Interleukin-4 (IL-4) for 72 hours.

Flow cytometric assessment of antibody binding to antigen and cell surface receptors

Binding of SF-25 IgE and IgG1 to Fc ϵ R- and Fc γ R-expressing cells or antigen-expressing cells was evaluated by using 10⁵ cells per sample. Cells were detached with PBS+5 mM EDTA, resuspended in PBS+2% FBS (FACS buffer) and incubated with 5 µg/ml of primary antibody for 30 minutes at 4°C, followed by a wash with 3 ml of FACS buffer (spinning at 500g for 5 minutes). Cells were incubated with the secondary antibody goat anti-human IgE-fluorescein isothiocyanate (FI-3040; Vector Laboratories) or goat anti-human IgG1-fluorescein

isothiocyanate (FI-3080; Vector Laboratories) at 30 µg/ml in FACS buffer for 30 minutes at 4°C, followed by one wash as above. Samples were resuspended in 100 µl of FACS buffer and analysed with a BD FACS Canto™ II.

Detection on endogenous IgE on differentiated macrophages

M0, M1 and M2 monocyte-derived (MDM) macrophages were detached with Accutase and 1×10^5 cells were aliquoted in FACS tubes in RPMI1640 medium supplemented with 2% FBS. Cells were incubated with 5 µg/ml of IgE antibody for 30 mins at 4°C. Macrophages were then washed with PBS to remove any unbound antibody. Cells were then treated with 1 µg/ml of goat polyclonal anti-human IgE–fluorescein isothiocyanate (FI-3040; Vector Laboratories) for 30 additional mins at 4°C. Cells were washed again with PBS by centrifugation for 5 mins at 400xg and resuspended in FACS buffer, before analysing them on a BD FACS Canto™ II.

IgE crosslinking on human macrophages

M0, M1 and M2 (MDM) macrophages were incubated with 5 µg/ml of IgE antibody for 30 mins at 37°C by adding the antibody solution directly onto the cell culture plate. Cells wells were washed with PBS to remove any unbound antibody. Cells were then treated with 1 µg/ml of goat polyclonal anti-human IgE–fluorescein isothiocyanate (FI-3040; Vector Laboratories) for 30 more mins at 37°C. Cells were harvested 4 hours after stimulation to examine soluble mediator release and 24 h after stimulation to investigate surface marker expression.

Magnetic Luminex Assay of macrophage supernatants for cytokine detection

Macrophage supernatants were collected 4 hours post-stimulation, centrifuged at 600 x g to remove floating cells and diluted 1:1 with sterile PBS. The multiplex assay was performed with the Magnetic Luminex Kit following the protocol instructions. The plate was sealed and left on the shaker up to 90 minutes until ready to be analysed on a Luminex™ analyzer.

Flow cytometry assessment of surface protein expression on macrophages

The day after the treatments, cells were detached with Accutase (Sigma). Plates were incubated for 10 minutes at 37°C to facilitate detachment. After the incubation, 1 ml of RPMI 1640 supplemented with 10% FCS and penicillin/streptomycin was added to each well. Cells were counted, washed with PBS by centrifugation at 400 x g for 5 minutes and resuspended at a concentration of 1×10^6 cells/ml. 5 ml poly-propylene tubes were used for staining and flow cytometry acquisition. To each sample, 1 µl of each of the staining antibody solution was added, and cells were incubated for staining at 4°C for 40 minutes, before being washed with 2 ml of FACS buffer and centrifuged as above. Cells were resuspended in 100 µl of PBS only and stained for live/dead cell discrimination with LIVE/DEAD® Fixable Dead Cell Stain Near-IR and incubated at room temperature for 30 minutes in the dark. Tubes were added with 2 ml of PBS and washed by centrifugation as above, before being resuspended in 200 µl of FACS buffer and acquired at BD FACS Canto™ II.

Investigation of gene expression via Real Time Quantitative PCR (RT-qPCR)

cDNA samples were diluted 1:4 with RNase/DNase free water and 2 µl were distributed in triplicate on a fast-optical 96-well reaction plate. A qPCR master mix was prepared by mixing 10 µl of TaqMan gene expression

master mix, 1 µl of GAPDH Probe, 1 µl of the GOI Probe (T cell (or transmembrane) immunoglobulin and mucin domain protein 3 *Tim-3* or *Lyn* kinase) and 6 µl RNase free water. The qPCR master mix was added to every well containing DNA. Negative qPCR controls included cDNA-free wells. 40 cycles of RT-qPCR were performed.

Cytotoxicity/Phagocytosis (ADCC/ADCP) Assay to study IgE antibody functions in activating macrophages against cancer cells

ADCC/ADCP assays were performed using a protocol adjusted from a previously-described method ⁽¹⁴⁾. Briefly, target tumour cells were labelled with Carboxyfluorescein succinimidyl ester (CFSE) the day before performing the assay. For this, cells were detached with trypsin and washed once in RPMI 1640 complete medium. Cells were then resuspended in 100 µl PBS, and 0.75 µl of 0.5 mM CFSE was added for every 1x10⁶ cells. Cells were incubated at 37°C for 10 minutes and the reaction was stopped with one wash in ice-cold RPMI 1640 standard medium. Normal culture conditions were resumed overnight. The following day, CFSE-labelled tumour cells were detached using PBS + 5mM EDTA, washed in RPMI 1640 standard medium and re-suspended to 1x10⁵ cells/100 µl culture medium. Macrophages were detached with Accutase (Sigma), re-suspended in culture medium and mixed with the tumour cells at a 10:1 ratio. Cells were placed in FACS tubes and co-incubated at 37°C for 3 hours in the presence of either PBS or 5 µg/ml of either SF-25 IgE or control antibody. After incubation, cells were washed in FACS buffer and macrophages were identified by staining mixed cell populations with 5 µg/ml of anti-human CD206-APC/Cy7-conjugated antibody to detect M1 or anti-human CD163-PE/Cy7-conjugated for M2 for 30 minutes at 4°C. Cells were washed once and re-suspended in 200 µl FACS buffer for immediate acquisition using a dual laser FACSCanto™ (Becton Dickinson). Every sample was treated with 100 µl of DAPI (100 ng/ml), mixed using a vortex and acquired immediately. Tumour cell killing was calculated on the percentage of DAPI⁺ tumour cells and the total number of tumour cells in SF-25 antibody-treated samples compared to negative controls. Analysis of flow cytometry data was performed by FlowJo (TreeStar Inc) software.

Analysis of the Phosphorylation profile of kinases

Proteome Profiler Human Phospho-Kinase Array (R&D Systems) were incubated with 400 µg lysate overnight at 4°C. The following day, chemiluminescent detection was performed according to manufacturer's indications. Densitometry analysis was conducted using ImageJ.

Study of FcεRI pathway in macrophages

Pathway analysis was performed using the Reactome Pathway Analysis tool ⁽¹⁵⁾. The set of genes of phosphorylated kinases used for input was the combined superset from those perturbed under both IgE stimulation of M1 and M2. In order to determine the ability of each gene product to discriminate between the observed differences in M1 and M2 phosphorylation, the difference in fold change between M1 and M2 was calculated. The M1/M2 difference in fold change was used to weight each gene when inferring implicated pathways. Kinases were considered “perturbed” by IgE-crosslinking when the t-test p-value performed on the means of each condition (baseline/IgE crosslinked), resulted in a P value of <0.05. A False Discovery Rate of less than 0.05 and a minimum gene set size of 4 was used to filter the results. Overly general pathways and processes were manually removed. Version 67 of the Reactome database was used. The KEGG database was used to depict the structure of the FcεRI pathway.

Statistical methods and analyses of publicly-available databases

All statistical analyses were performed using GraphPadTM Prism Software (version 6.0). Error bars represent SD and SEM in *in vitro* and in *ex vivo* evaluations. Clinical associations of gene expression were assessed using publicly-available data in Kaplan-Meier Plotter (<http://kmplot.com/analysis/>). One way-ANOVA was performed in combination with Bonferroni's or Tukey's post-test to determine statistical significance of the data.

Results

Human monocyte-derived macrophages express typical M1 and M2 differentiation surface markers and express IgE Fc receptors

To evaluate the impact of Fc engagement of IgE on human macrophage populations, we firstly confirmed the characteristics of classically-activated (M1) and alternatively-activated (M2) subsets ⁽²⁾. Human primary monocytes isolated from peripheral blood by negative selection and magnetic bead purification ^(16,17) comprised the three classical (CD14⁺⁺, CD16⁻), intermediate (CD14⁺, CD16⁺) and alternative (CD14⁻, CD16⁺⁺) subsets (Supplementary Figure 1A). Non-adherent, non-monocytic cells were removed after two hours of seeding in serum-free medium, consistently yielding > 80% monocyte purity based on CD14 and CD16 expression and physical parameters (Supplementary Figure 1A-B). Macrophages were differentiated over 7 days (Figure 1A), followed by cytokine stimulation (IFN γ +LPS for M1, IL-4 for M2) for 72 hours, to obtain classically (M1) and alternatively (M2) activated macrophage subsets, respectively. Consistent with previous reports ⁽¹⁸⁾, differentiated populations showed the expected morphology profiles: fried egg-shape like GM-CSF and M1 macrophages, and fibroblast-like, needle-shaped M-CSF macrophages, which further elongated after treatment with IL-4 (Figure 1A).

We ascertained expression of key markers of macrophage differentiation (co-stimulatory molecules CD80, CD86 for M1; the scavenger receptor cysteine-rich family member CD163 (or M130), and the mannose receptor CD206 for M2) (Figure 1B). Consistent with previous studies, peripheral blood monocytes expressed CD86 but not CD80 ⁽¹⁹⁾. A high proportion of M1 macrophages expressed both CD80 and CD86. We found expression of CD163 ⁽²⁰⁾ on 50% of freshly isolated monocytes and on a high proportion of alternatively-activated (M2) macrophages. Furthermore, 10% of primary monocytes and the majority of M1 and M2 macrophages expressed CD206, in line with previous reports ⁽²¹⁾. These findings confirmed the differentiation of classically- and alternatively-activated macrophage subsets from human monocytes.

Very little information is available regarding IgE receptor expression for distinct macrophage subpopulations ⁽¹³⁾. We found that 20% of peripheral blood monocytes expressed the high affinity IgE Fc receptor Fc ϵ RI, while 1-2% expressed the low affinity receptor Fc ϵ RII (CD23), in line with previously published studies ^(22,23) (Figure 1C). M2 macrophages displayed the highest levels of cell surface expression of Fc ϵ RI (40% of cells, n=4), while CD23 expression was expressed by 15-20% of all macrophage phenotypes (M0, M1, M2).

Based on the high affinity of IgE for cognate Fc receptors and its slow dissociation rates most notably following binding to Fc ϵ RI on immune cells, we hypothesised that some endogenous IgE may be retained by effector cells *ex vivo* in the absence of antigen engagement and immune complex formation ^(24,25). An anti-human IgE FITC-conjugated antibody was used to detect endogenous IgE and also following treatment with anti-tumour IgE (SF-25) to understand whether free IgE Fc receptors were available for exogenous IgE to bind and, to crosslink these on the surface of macrophages ⁽²⁶⁻²⁸⁾. Over 20% of M0 macrophages were detected to still have cell surface-bound endogenous IgE when incubated with anti-IgE antibody alone (Supplementary Figure 2). Both M1 and M2 macrophages showed detectable but lower endogenous IgEs on their surface. All cell subsets could also bind exogenous IgEs.

Together, as previously demonstrated with primary human monocytes ^(29,30), these findings indicate that IgE Fc receptors were not saturated on the surface of human macrophages and that exogenous IgEs could still engage with these cognate Fcε receptors despite the presence of endogenously-bound IgE.

IgE crosslinking on macrophages upregulates M1 surface marker CD80 levels and triggers secretion of a distinct cytokine chemokine signature by M0 and M2 macrophages

We previously reported that systemic administration of rat anti-tumour IgE was associated with recruitment of CD80+ macrophages into syngeneic tumours of immunocompetent rats ⁽⁹⁾. Here we investigated whether IgE stimulation might impact on human classically and alternatively activated macrophage surface marker expression. Human macrophage subsets were incubated with either anti-tumour (SF-25) IgE or the hapten-specific antibody NIP IgE. IgE-Fcε receptor crosslinking was triggered by polyclonal anti-human IgE. Crosslinking of IgE on macrophages did not affect CD14, FcεRI or CD23 surface expression, while FcγRIII (CD16) expression by M2 macrophages was reduced with crosslinking of both SF-25 and NIP IgE (Supplementary Figure 2). Surface expression of CD80 was upregulated on M0 and M2 macrophages when cell bound IgE was crosslinked but remained unaltered on M1 macrophages. Neither IgE engagement nor crosslinking affected CD163 or CD206 surface expression on any of the cell subsets.

Based on previous evidence that IgE can influence macrophage recruitment and activation in rodent models ⁽⁹⁾, we investigated whether IgE crosslinking of Fcε receptors on the surface of different human macrophage subsets could influence soluble mediator release (Figure 2A):

Pro-inflammatory cytokines TNFα, IFNγ, IL-12, IL-1β: Crosslinking of IgE led to a drastic increase (from 100 to approximately 2,000 pg/ml) in TNFα secretion in both M0 and M2 macrophages, while IgE did not affect the low TNFα levels produced by M1 cells (Figure 2B). IgE crosslinking enhanced IFNγ secretion by both M0 and M2 subsets. M1 macrophages showed a 4-fold higher baseline levels of IFNγ compared to the other subsets and these remained stable upon activation via IgE. Furthermore, M0 and M2 macrophages increased IL-12 production from 100 to approximately 1500-2000 pg/ml when crosslinked with IgE/anti-IgE. M1 cells featured a greater baseline level of IL-12 (ca. 1,500 pg/ml), which remained unaffected upon IgE treatment. Baseline concentration of IL-1β were very low in all three cell subsets. Crosslinking of cell-bound IgE triggered increased IL-1β secretion in M0 and M2 macrophages, while stimulation did not influence production by M1 macrophages.

Anti-inflammatory cytokines IL-4, IL-6, IL-10, IL-13: IL-4 production slightly increased upon IgE crosslinking in both M0 and M2 subsets and remained unaffected in M1 cells. Baseline levels of IL-10 and IL-13 were similar between macrophage subsets, however IgE crosslinking upregulated IL-10 and IL-13 release by M0 and M2 subsets but not by M1 macrophages. IL-6 secretion remained unchanged in M1 macrophages but increased upon treatment with IgE only and with IgE/anti-IgE crosslinking in both M0 and M2 subsets (Figure 2C).

Interferon Gamma-induced chemokines MIG (CXCL9) and I-TAC (CXCL11): In M1 macrophages, secretion of MIG (CXCL9) and I-TAC (CXCL11) remained unchanged (ca. 400 pg/ml) with IgE stimulation, while crosslinking of IgE in M0 and M2 macrophages led to modest increases. Levels of I-TAC (CXCL11) were 4-fold greater in M1 macrophages (ca. 400 pg/ml) compared to M0 and M2 at baseline, however, IgE crosslinking increased secretion of I-TAC (CXCL11) by M0 and M2 cultures from ca. 80 to ca. 160 pg/ml in M0 cells and from 50 to ca. 200 pg/ml in M2 macrophages (Figure 2D).

Macrophage chemoattractant chemokines MCP-1 (CCL2) and RANTES (CCL5): In a rat model of cancer and in human monocytes *in vitro*, Josephs and colleagues demonstrated, that IgE crosslinking triggered a TNF α /MCP-1 axis, which resulted in recruitment of macrophages into tumours ⁽⁹⁾. Here, crosslinking of cell-bound IgE produced no modulation of MCP-1 supernatant concentration in either M0 or M1 macrophages. However, M2 macrophages, responded to IgE activation by enhancing the release of MCP-1. M0 and M2 but not M1 macrophages increased production of RANTES with IgE crosslinking, with the peak concentrations following IgE stimulation corresponding approximately to the baseline concentration in M1 cells (Figure 2E).

Together these data demonstrate that crosslinking of cell-bound IgE triggered enhanced expression of cytokines TNF α , IFN γ , IL-12, IL-1 β , IL-6, IL-10, IL-13 and of the chemokines MIG (CXCL9), I-TAC (CXCL11) and RANTES (CCL5) by M2 and M0 macrophages. IgE crosslinking on M2 macrophages but not on M0 or M1 subsets, triggered enhanced secretion of the chemoattractant MCP-1 (CCL2). On the other hand, M1 macrophages appeared to retain their cytokine/chemokine expression upon IgE activation including expression of pro-inflammatory mediators such as IFN γ and IL-12 (Table 1).

Crosslinking of IgE on M1 and M2 macrophages regulates phosphorylation levels of protein kinases downstream of Fc ϵ RI signalling pathway

Crosslinking of differentiated macrophages M1 and M2 with IgE/anti-IgE resulted in modification of the phosphorylation levels of intracellular kinases, analysed via a phosphorylation array (Figure 3A). Amongst 43 kinases analysed, overall 29 were significantly perturbed in IgE-stimulated macrophages. M2 macrophages were characterised by a marked increase in phosphorylation levels compared to the M1 subset, which in contrast displayed a decrease of phosphorylation for the majority of kinases analysed (Figure 3B). M2 macrophages displayed a marked increase (1.25-5-fold changes) in the phosphorylation of HSP27, p38, p27, ERK1/2 and Lck kinases following crosslinking. Crosslinking of IgE on M1 macrophages induced downregulation of p38, ERK1/2, STAT5a/b, GSK3a/b, Chk2 and FAK phosphorylation, while some kinases showed upregulated phosphorylation of maximum 0.2-fold change compared to baseline (Figure 3C). MSK1/2 was the only kinase displaying slightly increased phosphorylation of 0.2-fold change in both macrophage subsets (Figure 3C).

In order to investigate whether IgE stimulation influenced Fc ϵ RI signalling, commonly known to be activated on mast cells, we interrogated the publicly-available KEGG database to depict the Fc ϵ RI signalling pathway. Molecular species whose phosphorylation level was measured *ex vivo* from IgE-stimulated differentiated human macrophages are depicted in blue (Figure 4A). Since the Fc ϵ RI signalling pathway in human macrophages is relatively unexplored, we interrogated a publicly available database (Reactome) with the proteome phospho-profiler dataset to dissect differences between M1 and M2 macrophage signalling cascades upon IgE crosslinking. IgE crosslinking on macrophages triggered phosphorylation of kinases known in mast cells to be found downstream of the Fc ϵ RI pathway network (generated via the KEGG database) (Figure 4A). Furthermore, a number of signalling pathways appeared to be differentially activated following IgE crosslinking in the two macrophage subsets (VEGFA-VEGFR2 pathway, PI3K/AKT and Rho GTPases signalling amongst others), with the MAPK family pathway being the most differentiating between classically- and alternatively-activated macrophages (Figure 4B). These findings confirm for the first time that IgE stimulation potentiates activation of kinases downstream of the Fc ϵ RI signalling pathway in macrophages and reflect differential intracellular

mechanisms specific for each alternatively-activated and classically-macrophage subset triggered by activation of FcεRI.

IgE crosslinking on macrophages modulates gene expression of *Lyn* and *Tim-3* and *Lyn* gene expression is associated with a favourable prognosis in solid tumours

We next investigated the gene expression of the protein kinase *Lyn* and the checkpoint molecule *Tim-3* (T cell or transmembrane immunoglobulin and mucin domain protein 3), both known to participate in the FcεRI signalling pathway in mast cells ⁽³¹⁾. Upon IgE crosslinking, *Lyn* mRNA expression increased to 2-, 7- and 70-fold in M0, M1 and M2 macrophages, respectively (Figure 5A, left). M2 macrophages downregulated *Tim-3* expression upon IgE activation, while M0 macrophages increased *Tim-3* gene expression by 150-fold in response to IgE crosslinking compared to untreated cells (Figure 5A, right). Together these data demonstrate that the FcεRI pathway can be activated with IgE on macrophage subsets *ex vivo* and gene expression of *Lyn* and *Tim-3* are differentially regulated by IgE on distinct macrophage subsets.

To gain insights into a potential clinical relevance of IgE/FcεR signalling through *Lyn* and *Tim-3*, we interrogated publicly-available gene expression datasets for solid tumours of different origins (Figure 5B). We found significant association of improved 5-year overall survival with elevated expression of *Lyn* in gastric ($P=7.7e-08$), lung ($P=6.7e-05$) and ovarian ($P=0.036$) cancers. These findings suggest that enhanced *Lyn* gene expression - one of the downstream signalling effects of IgE crosslinking its FcεR on alternatively-activated and classically-activated macrophages - may be associated with better prognosis in cancer patients. On the other hand, the association of *Tim-3* gene expression with clinical outcomes appeared dependent on the tumour type: specifically, higher levels of *Tim-3* expression in lung and ovarian cancers correlated with improved patient prognosis, while it was associated with less favourable overall survival in patients with gastric cancer. This suggests that *Tim-3* may exert different functions in different malignancies. These findings may be significant in future IgE class-mediated immune cell activation in different solid tumour types.

M0, M1 and M2 derived human macrophages can be activated by anti-tumour IgE to kill cancer cells

We assessed whether human macrophages derived *ex vivo* can be activated by IgE to kill cancer cells by applying a three-colour flow cytometric assay to detect cytotoxicity and phagocytosis (14) (Figure 6A). The anti-tumour antibody SF-25 IgE was successful in engaging all three macrophage subsets, M0, M1 and M2 to mediate tumour antigen-specific ADCC of A-2058 cancer cells compared with non-specific antibody-treated or no antibody control cells (Figure 6B). SF-25 IgE triggered approximately 30% of tumour cell-killing above controls. On the other hand, treatment of macrophages and A-2058 tumour cells with SF-25 IgG1 isotype antibody did not produce effective tumour cell killing above isotype controls. These findings support the notion that IgE antibodies can activate human macrophages to kill tumour cells *ex vivo*, but also that both M1 and M2 subsets can engage with antibodies of the ε class and target cancer cells by exerting antigen-specific cytotoxic activity.

In summary, crosslinking IgE antibodies on the surface of human macrophages influenced their polarisation status (Figure 7), leading quiescent macrophages to acquire M1-like features, M1 macrophages to retain their pro-inflammatory status and skewing differentiated M2 macrophages towards a pro-inflammatory phenotype, with measurable anti-tumour activity when tumour antigen-specific IgE could target macrophages against cancer cells.

Discussion

Macrophages represent a major component of the tumour microenvironment in many solid cancers. Their phenotypes and activation states can be influenced in the TME in response to environmental stimuli, so that different macrophage subsets could orchestrate different and often opposite aspects of the immune response, striking a balance between anti-tumour immunity and wound healing or inflammation. The potential of antibodies to influence the dynamics and polarisation states of these cells against cancer may offer opportunities to tip the balance in favour of host immunity. However, the mechanisms of human macrophage-associated immune complex formation engendered by different antibody isotypes, and especially through the IgE/FcεR (IgE FcR) axis, remain insufficiently understood.

We have previously reported that systemic treatment with anti-tumour IgE reduced tumour growth in different *in vivo* models of cancer, associated with significant recruitment of macrophages towards tumours ^(9,29). However, the mechanisms by which therapeutic antibodies of the IgE class can engage, influence and activate tissue macrophage populations, especially those likely to form part of the tumour microenvironment, remain unexplored. In this study we investigated whether IgE stimulation may influence the polarisation and activation states of classically- and alternatively-activated macrophage populations. For this purpose, we derived human macrophages displaying the typical markers of M1 (classically-activated) or M2 (alternatively-activated) phenotypes and cytokine profiles ⁽³²⁾. We therefore explored the presence of cognate Fcε receptors on the surface of classically- and alternatively-activated macrophages and interrogated the effect of IgE engagement and of crosslinking of macrophage-bound IgE on each subset.

Around 40% of M2 macrophages expressed the high affinity IgE receptor FcεRI, while approximately 20% of cells in each subset expressed the low affinity IgE receptor CD23. Macrophage cell surface-bound IgE crosslinked on alternatively-activated macrophages triggered increased expression of the co-stimulatory molecule CD80, a typical M1 macrophage marker. Upregulation of the co-stimulatory molecule CD80 on the surface of professional antigen-presenting cells (APC) including macrophages, may signify cell maturation and also confer capacity for effective antigen presentation and may point to the potential of IgE class antibodies to promote macrophage maturation, polarisation and APC functions. These findings are consistent with previously described evidence of: a) infiltration of immune cells, including macrophages, in human ovarian cancer patient-derived xenograft residual tumours in immunocompromised mice treated with an anti-tumour IgE and human PBMCs as effector cells; and b) higher recruitment of monocyte/macrophages into tumours and enhanced expression of CD80+ rat macrophages into syngeneic tumours in an immunocompetent rat model following systemic treatment with rat MOv18 IgE ^(4,9). Furthermore, in our study, IgE stimulation did not interfere with the expression of CD163 on either macrophage population. In concordance, we previously reported that in rats treated with anti-tumour IgE, tumour-infiltrating rat macrophages did not upregulate the M2 marker CD163 ⁽⁹⁾. Together, these indicate that activation of the IgE/FcεR pathway may influence macrophage phenotype and maturation.

We furthermore explored whether IgE stimulation may promote differential activation of specific macrophage subsets. In M1 macrophages, IgE crosslinking retained the production of key pro-inflammatory cytokines including IFNγ and IL-12. In concordance, in Josephs et al, we recently demonstrated upregulation of pro-inflammatory immune-associated pathways including IL-12 and NK-cell immune activation signatures in the lungs of tumour-bearing rats treated with MOv18 IgE ⁽¹⁰⁾. Considering these findings, it is possible that IgE

engagement and crosslinking may at least retain M1 macrophages in a manner consistent with their known pro-inflammatory and antigen-presenting functions.

Importantly, secretion of IL-1 β , IL-6, IL-10, IL-12, IL-13, IFN γ , RANTES, MIG and I-TAC was triggered by crosslinking of cell-bound IgE on quiescent M0 and on M2 macrophages. This may suggest that the two subsets might feature similar molecular mechanisms in their ability to regulate activation of the Fc ϵ RI pathway. Furthermore, production of all soluble mediators tested was either retained at the same level or increased with IgE-crosslinking. Although the cytokine panel secreted by M0 and M2 after crosslinking with IgE did not correspond to a defined macrophage subtype, the mediator signature displayed by IgE-stimulated cells may represent a newly polarised macrophage subset secreting both pro- and anti-inflammatory mediators, as well as chemoattractant factors (Table 1). An additional result that also points at the development of a macrophage state with activatory features is increased release of CXCL9 with IgE stimulation in M0 and M2 macrophages: CXCL9 represents an important anti-angiogenic factor, known to be predominantly expressed by the M1 phenotype ⁽³³⁾. Since CXCL9 is a direct counter-regulatory mediator of VEGF signalling known to influence macrophage polarisation ⁽³⁴⁾, future studies may also directly assess the impact of IgE antibody stimulation on the expression of VEGF by different macrophage subsets.

We also report that IgE crosslinking on both M0 and M2 macrophages triggered enhanced levels of the pro-inflammatory M1 cytokine TNF α , which this had different effects in M0 and M2 macrophages in the relation to the production of the macrophage chemoattractant MCP-1. M2 macrophages, upon IgE treatment, enhanced the production and the release of MCP-1. In line with the findings presented in this work, upregulation and the potential roles of TNF α and MCP-1 have been described by Josephs and colleagues. Our previous findings with the anti-tumour IgE antibody MOv18 in a rat tumour model *in vivo* suggested that tumour antigen-specific IgE treatment influences the spatial distribution and activation of macrophages around tumour lesions. Systemic IgE treatment in this model was also associated with a specific cytokine signature, featuring elevated levels of TNF α , MCP-1 and IL-10 in the tumour microenvironment and upregulated expression of TNF α by tumour-associated macrophages in rat lungs. Furthermore, *ex vivo* crosslinking of MOv18 IgE, triggered TNF α upregulation by human monocytes. TNF α stimulation triggered production of MCP-1 by macrophages and also by tumour cells of different origins. MCP-1 triggered monocyte and macrophage infiltration in the tumour microenvironment. These studies demonstrated the significance of the TNF α /MCP-1 axis in the modulation of cellular populations and functions in the TME ^(9,35). Our findings that M0 and M2 macrophages can be stimulated to secrete TNF α and that alternatively activated M2 macrophages are the only cell type that upregulated MCP-1 upon crosslinking by IgE, suggest that IgE has the capacity to specifically stimulate this normally anti-inflammatory subset to a more mature, further activated phenotype, which bears some characteristics normally associated with the classically-activated M1 macrophages. Further investigation of the effects of IgE antibodies on the expression of additional soluble mediators such as IL-17A, and other family members, and IL-37, reported to influence macrophage phenotype, may shed light into the impact of IgE-Fc ϵ Rs interactions in the tumour microenvironment ^(36,37).

Analysis of gene expression of the protein kinase *Lyn* and *Tim-3* both part of the Fc ϵ RI signalling pathway and known to regulate effector cell activation ⁽³⁸⁾, showed that crosslinking of IgE on macrophages upregulated *Lyn* gene expression in M0, M1 and M2 macrophage subtypes. This may be significant in the cancer context since *in vivo* studies demonstrated that a loss-of-function of *Lyn* may predispose to tumorigenesis ⁽³⁹⁾, and transgenic mouse models lacking *Lyn* were characterised by defective macrophage populations ⁽⁴⁰⁾. The retention and

upregulation of *Lyn* expression on all macrophage subsets may therefore signify the importance of activating macrophages by antibodies of the IgE isotype, which could potentially confer protection from tumour growth. The potential of activating the FcεR downstream pathway, and by inference anti-tumour IgE engagement of immune effector cells may have clinical significance, since higher intra-tumoural *Lyn* expression in gastric, lung and ovarian cancer is associated with more favourable prognosis. On the other hand, engagement and crosslinking of IgE induced a reduction in *Tim-3* gene expression in M2 macrophages, while *Tim-3* was upregulated in M0 macrophages. *Tim-3*, known to participate in multiple suppressor pathways of anti-tumour responses, can be expressed by cancer cells and may support alternatively-activated macrophage polarisation. Simultaneous blocking of *Tim-3* and PD-1 in infectious diseases was shown to restore lymphocyte functions and it was recently hypothesised that blocking *Tim-3* may support the anti-tumour functions by macrophages ⁽⁴¹⁾. IgE crosslinking and subsequent reduction in *Tim-3* gene expression in M2 macrophages may induce immune cell activation, in line with what was observed with T lymphocytes upon treatment with anti-PD-1 and anti-*Tim-3* antibodies. Therefore, reduction of *Tim-3* expression on M2 macrophages upon IgE treatment may be further explored in the context of cancer immunotherapy and macrophage functions in the TME.

In summary, analysing surface marker expression, soluble mediator release, gene expression and protein phosphorylation on human macrophages derived *ex vivo* upon IgE crosslinking may provide a new insight on this antibody class that may have relevance in tissues and perhaps in TME. Quiescent M0 macrophages, when crosslinked with IgE, featured: upregulation of the classic M1 surface marker CD80, increased production of pro-inflammatory cytokines and chemoattractant mediators, increased expression of the kinase *Lyn* and other downstream signalling molecules including Erk1/2 and p38. M1 macrophages retained their pro-inflammatory status upon activation via IgE antibodies, which led to increased expression of *Lyn* downstream of the FcεRI pathway. The crosslinking of IgE antibodies on M2 macrophages was characterised by a significant increase in the production of the chemoattractant, pro-inflammatory molecule MCP-1 and the enhanced phosphorylation and gene expression of *Lyn*. Therefore, crosslinking of IgE on human macrophages confers M0 and M2 subsets features more likely associated with classically-activated macrophages (Figure 7), leading to polarised macrophage phenotypes. Our functional assays indicate that anti-tumour IgE antibodies can engage human macrophages to elicit an effector function response against cancer cells. We show for the first time that IgE can activate macrophages of different polarisation states to kill cancer cells. This supports the notion that tissue type macrophages, including those subsets commonly associated with tumour lesions, could be engaged and re-educated by anti-tumour IgE antibodies, and importantly, that IgE could unleash their cytotoxic potential against target tumour cells.

The anti-cancer potential of IgE class antibodies has been reported in multiple studies in the emerging field of AllergoOncology, concerned with the links between Th2 and IgE responses with cancer ^(42,43). Reports include an IgE antibody recognising the cell surface tumour antigen HER2/*neu* which restricted tumour growth in human FcεRI transgenic mice. Furthermore, Fazekas-Singer et al. reported the development of a canine IgE targeting the epidermal growth factor receptor (EGFR) shown to engage and activate canine macrophages more effectively than the equivalent IgG1 to target and kill tumour cells *in vitro* ⁽⁴⁴⁾. Human adipose tissue-derived mast cells (ADMC) can be specifically activated to display tumoricidal activity against breast cancer cells when engaged with tumour antigen-specific IgEs ⁽⁴⁵⁾. Our own studies have demonstrated the capacity of an anti-FRα IgE antibody (MOv18) to restrict tumour growth in several model systems and to recruit monocytes and macrophages

against tumours, supporting the progress of this agent to a first-in-class Phase I clinical trial in oncology (NCT02546921, www.clinicaltrials.gov).

Taken together, our findings point to the potential of anti-tumour IgE antibodies not only to mediate immune effector cell-mediated killing of cancer cells, but also to impact on immune stroma, influencing macrophage phenotype and maturation states. Our findings provide new insights into the interactions between human macrophages and IgE class antibodies. This may be highly significant in the clinical development of new immunotherapy strategies targeting these often tissue- and tumour-resident cells and may point to previously-unappreciated cascades with potential to activate and re-educate immune stroma.

Acknowledgments

We acknowledge the Biomedical Research Centre Immune Monitoring Core Facility and the Biomedical Research Centre Genomics Research Platform teams at Guy's and St Thomas' NHS Foundation Trust.

Funding sources

The authors acknowledge support by Breast Cancer Now (147), working in partnership with Walk the Walk; Cancer Research UK (C30122/A11527; C30122/A15774); the Medical Research Council (MR/L023091/1); the Academy of Medical Sciences; the Inman Charity; CR UK//NIHR in England/DoH for Scotland, Wales and Northern Ireland Experimental Cancer Medicine Centre (C10355/A15587); CR UK/EPSRC/MRC/NIHR KCL/UCL Comprehensive Cancer Imaging Centre (C1519/A10331). The research was supported by the National Institute for Health Research (NIHR) Biomedical Research Centre (BRC) based at Guy's and St Thomas' NHS Foundation Trust and King's College London (IS-BRC-1215-20006). The authors are solely responsible for study design, data collection, analysis, decision to publish, and preparation of the manuscript. The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR or the Department of Health.

Declaration of Interests

S.N. Karagiannis and J.F. Spicer are founders and shareholders of IGEM Therapeutics Ltd. S.N. Karagiannis holds a patent on anti-tumour IgE antibodies. All other authors have declared that no conflict of interest exists.

Author contributions

GP, SNK, DHJ, JFS and HJB conceived the study; GP conceived, performed the experiments, analysed the data and coordinated the study; CH performed the pathway analysis; ST conceived and supervised the bioinformatics analyses; SC, SM and JG contributed to data collection and analysis; GC, RB, HJB, KI contributed to data interpretation. SP, ST, KEL, DHJ, JFS, EJJ, SNK provided guidance and discussed the manuscript. SNK led and supervised the study. All authors approved the final version of the manuscript.

References

1. Sica A, Larghi P, Mancino A, Rubino L, Porta C, Totaro MG, et al. Macrophage polarization in tumour progression. *Semin Cancer Biol.* 2008;18(5):349–55.
2. Aras S, Zaidi MR. TAMEless traitors: macrophages in cancer progression and metastasis. *Br J Cancer* [Internet]. 2017 Nov 21 [cited 2018 Jun 25];117(11):1583–91. Available from: <http://www.nature.com/doi/10.1038/bjc.2017.356>
3. Karagiannis SN, Josephs DH, Bax HJ, Spicer JF. Therapeutic IgE Antibodies: Harnessing a Macrophage-Mediated Immune Surveillance Mechanism against Cancer. *Cancer Res* [Internet]. 2017 Jun 1 [cited 2018 Jul 16];77(11):2779–83. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/28526770>
4. Josephs DH, Bax HJ, Lentfer H, Selkirk C, Spicer JF, Karagiannis SN. Potential for monocyte recruitment by IgE immunotherapy for cancer in a rat model of tumour metastasis. *Lancet* [Internet]. 2015 Feb 26 [cited 2018 Apr 21];385:S53. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/26312875>
5. Petrillo M, Zannoni GF, Martinelli E, Pedone Anchorà L, Ferrandina G, Tropeano G, et al. Polarisation of Tumor-Associated Macrophages toward M2 Phenotype Correlates with Poor Response to Chemoradiation and Reduced Survival in Patients with Locally Advanced Cervical Cancer. Rota R, editor. *PLoS One* [Internet]. 2015 Sep 3 [cited 2018 Jun 25];10(9):e0136654. Available from: <http://dx.plos.org/10.1371/journal.pone.0136654>
6. Zhang M, He Y, Sun X, Li Q, Wang W, Zhao A, et al. A high M1/M2 ratio of tumor-associated macrophages is associated with extended survival in ovarian cancer patients. *J Ovarian Res* [Internet]. 2014 Feb 8 [cited 2018 Jun 25];7:19. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/24507759>
7. Poh AR, Ernst M. Targeting Macrophages in Cancer: From Bench to Bedside. *Front Oncol* [Internet]. 2018 Mar 12 [cited 2018 Jul 18];8:49. Available from: <http://journal.frontiersin.org/article/10.3389/fonc.2018.00049/full>
8. Georgoudaki AM, Prokopec KE, Boura VF, Hellqvist E, Sohn S, Östling J, et al. Reprogramming Tumor-Associated Macrophages by Antibody Targeting Inhibits Cancer Progression and Metastasis. *Cell Rep.* 2016;15(9):2000–11.
9. Josephs DH, Bax HJ, Dodev T, Georgouli M, Nakamura M, Pellizzari G, et al. Anti-Folate Receptor- α IgE but not IgG Recruits Macrophages to Attack Tumors via TNF α /MCP-1 Signaling. *Cancer Res* [Internet]. 2017 Mar 1 [cited 2018 Jun 22];77(5):1127–41. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/28096174>
10. Josephs DH, Nakamura M, Bax HJ, Dodev TS, Muirhead G, Saul L, et al. An immunologically relevant rodent model demonstrates safety of therapy using a tumour-specific IgE. *Allergy* [Internet]. 2018 Dec [cited 2019 Feb 3];73(12):2328–41. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/29654623>
11. Kalesnikoff J, Huber M, Lam V, Damen JE, Zhang J, Siraganian RP, et al. Monomeric IgE stimulates

- signaling pathways in mast cells that lead to cytokine production and cell survival. *Immunity* [Internet]. 2001 Jun 1 [cited 2019 Jan 9];14(6):801–11. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/11420049>
12. Gallenga CE, Pandolfi F, Caraffa A, Kritas SK, Ronconi G, Toniato E, et al. Interleukin-1 family cytokines and mast cells: activation and inhibition. *J Biol Regul Homeost Agents* [Internet]. [cited 2019 Mar 21];33(1):1–6. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/30656901>
 13. Clanchy FIL. High-Affinity Fc Receptor Expression Indicates Relative Immaturity in Human Monocytes. [cited 2018 Jun 5]; Available from: <https://www.liebertpub.com/doi/pdf/10.1089/jir.2015.0060>
 14. Bracher M, Gould HJ, Sutton BJ, Dombrowicz D, Karagiannis SN. Three-colour flow cytometric method to measure antibody-dependent tumour cell killing by cytotoxicity and phagocytosis. *J Immunol Methods* [Internet]. 2007 Jun 30 [cited 2018 Apr 20];323(2):160–71. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/17531261>
 15. Fabregat A, Jupe S, Matthews L, Sidiropoulos K, Gillespie M, Garapati P, et al. The Reactome Pathway Knowledgebase. *Nucleic Acids Res* [Internet]. 2018 Jan 4 [cited 2019 Jan 31];46(D1):D649–55. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/29145629>
 16. Sprangers S, Vries TJ de, Everts V. Monocyte Heterogeneity: Consequences for Monocyte-Derived Immune Cells. *J Immunol Res* [Internet]. 2016 Jul 11 [cited 2018 May 28];2016:1–10. Available from: <http://www.hindawi.com/journals/jir/2016/1475435/>
 17. Herter S, Birk MC, Klein C, Gerdes C, Umana P, Bacac M. Glycoengineering of Therapeutic Antibodies Enhances Monocyte/Macrophage-Mediated Phagocytosis and Cytotoxicity. *J Immunol* [Internet]. 2014 Mar 1 [cited 2019 Feb 9];192(5):2252–60. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/24489098>
 18. McWhorter FY, Wang T, Nguyen P, Chung T, Liu WF. Modulation of macrophage phenotype by cell shape. *Proc Natl Acad Sci U S A* [Internet]. 2013 Oct 22 [cited 2018 Jun 7];110(43):17253–8. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/24101477>
 19. Fleischer J, Soeth E, Reiling N, Grage-Griebenow E, Flad HD, Ernst M. Differential expression and function of CD80 (B7-1) and CD86 (B7-2) on human peripheral blood monocytes. *Immunology* [Internet]. 1996 Dec [cited 2018 Jun 5];89(4):592–8. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/9014827>
 20. Buechler C, Ritter M, Orsó E, Langmann T, Klucken J, Schmitz G. Regulation of scavenger receptor CD163 expression in human monocytes and macrophages by pro- and antiinflammatory stimuli. *J Leukoc Biol* [Internet]. 2000 Jan [cited 2018 Jun 6];67(1):97–103. Available from: <http://doi.wiley.com/10.1002/jlb.67.1.97>
 21. Alves-Januzzi AB, Brunialti MKC, Salomao R. CD163 and CD206 expression does not correlate with tolerance and cytokine production in LPS-tolerant human monocytes. *Cytom Part B Clin Cytom* [Internet]. 2017 May [cited 2018 Jun 6];92(3):192–9. Available from:

<http://www.ncbi.nlm.nih.gov/pubmed/26352275>

22. Alderson MR, Tough TW, Ziegler SF, Armitage RJ. Regulation of human monocyte cell-surface and soluble CD23 (Fc epsilon RII) by granulocyte-macrophage colony-stimulating factor and IL-3. *J Immunol* [Internet]. 1992 Aug 15 [cited 2019 Feb 9];149(4):1252–7. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/1386861>
23. Karagiannis SN, Bracher MG, Beavil RL, Beavil AJ, Hunt J, McCloskey N, et al. Role of IgE receptors in IgE antibody-dependent cytotoxicity and phagocytosis of ovarian tumor cells by human monocytic cells. *Cancer Immunol Immunother* [Internet]. 2007 Nov 19 [cited 2018 Jun 19];57(2):247–63. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/17657488>
24. Sutton B, Davies A, Bax H, Karagiannis S, Sutton BJ, Davies AM, et al. IgE Antibodies: From Structure to Function and Clinical Translation. *Antibodies* [Internet]. 2019 Feb 22 [cited 2019 Mar 25];8(1):19. Available from: <http://www.mdpi.com/2073-4468/8/1/19>
25. Holdom MD, Davies AM, Nettleship JE, Bagby SC, Dhaliwal B, Girardi E, et al. Conformational changes in IgE contribute to its uniquely slow dissociation rate from receptor FcεRI. *Nat Struct Mol Biol* [Internet]. 2011 May 24 [cited 2019 Mar 25];18(5):571–6. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/21516097>
26. Gould HJ, Mackay GA, Karagiannis SN, O'Toole CM, Marsh PJ, Daniel BE, et al. Comparison of IgE and IgG antibody-dependent cytotoxicity in vitro and in a SCID mouse xenograft model of ovarian carcinoma. *Eur J Immunol* [Internet]. 1999 Nov [cited 2018 Jul 27];29(11):3527–37. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/10556807>
27. Dodev TS, Karagiannis P, Gilbert AE, Josephs DH, Bowen H, James LK, et al. A tool kit for rapid cloning and expression of recombinant antibodies. *Sci Rep* [Internet]. 2014 Jul 30 [cited 2018 Mar 7];4:5885. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/25073855>
28. Crescioli S, Chiaruttini G, Mele S, Ilieva KM, Pellizzari G, Spencer DIR, et al. Engineering and stable production of recombinant IgE for cancer immunotherapy and AllergoOncology. *J Allergy Clin Immunol* [Internet]. 2018 Jan 31 [cited 2018 Mar 31];0(0). Available from: <http://www.ncbi.nlm.nih.gov/pubmed/29360527>
29. Karagiannis SN, Wang Q, East N, Burke F, Riffard S, Bracher MG, et al. Activity of human monocytes in IgE antibody-dependent surveillance and killing of ovarian tumor cells. *Eur J Immunol* [Internet]. 2003 Apr [cited 2018 Jul 15];33(4):1030–40. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/12672069>
30. Karagiannis SN, Bracher MG, Hunt J, McCloskey N, Beavil RL, Beavil AJ, et al. IgE-antibody-dependent immunotherapy of solid tumors: cytotoxic and phagocytic mechanisms of eradication of ovarian cancer cells. *J Immunol* [Internet]. 2007 Sep 1 [cited 2018 Jul 23];179(5):2832–43. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/17709497>
31. Phong BL, Avery L, Sumpter TL, Gorman J V., Watkins SC, Colgan JD, et al. Tim-3 enhances FcεRI-proximal signaling to modulate mast cell activation. *J Exp Med* [Internet]. 2015 Dec 14 [cited 2019 Feb

- 3];212(13):2289–304. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/26598760>
32. Martinez FO, Gordon S. The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000Prime Rep* [Internet]. 2014 [cited 2018 Jul 17];6:13. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/24669294>
33. Mantovani A, Sozzani S, Locati M, Allavena P, Sica A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol* [Internet]. 2002 Nov 1 [cited 2019 Mar 25];23(11):549–55. Available from: <https://www.sciencedirect.com/science/article/pii/S1471490602023025?via%3Dihub>
34. Riabov V, Gudima A, Wang N, Mickley A, Orekhov A, Kzhyshkowska J. Role of tumor associated macrophages in tumor angiogenesis and lymphangiogenesis. *Front Physiol* [Internet]. 2014 [cited 2019 Mar 21];5:75. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/24634660>
35. Josephs DH, Nakamura M, Bax HJ, Dodev TS, Muirhead G, Saul L, et al. An immunologically relevant rodent model demonstrates safety of therapy using a tumour-specific IgE. *Allergy*. 2018 Dec;73(12):2328–41.
36. Nakai K, He Y-Y, Nishiyama F, Naruse F, Haba R, Kushida Y, et al. IL-17A induces heterogeneous macrophages, and it does not alter the effects of lipopolysaccharides on macrophage activation in the skin of mice. *Sci Rep* [Internet]. 2017 Dec 29 [cited 2019 Mar 21];7(1):12473. Available from: <http://www.nature.com/articles/s41598-017-12756-y>
37. Nold MF, Nold-Petry CA, Zepp JA, Palmer BE, Bufler P, Dinarello CA. IL-37 is a fundamental inhibitor of innate immunity. *Nat Immunol* [Internet]. 2010 Nov 10 [cited 2019 Mar 21];11(11):1014–22. Available from: <http://www.nature.com/articles/ni.1944>
38. Phong BL, Avery L, Sumpter TL, Gorman J V., Watkins SC, Colgan JD, et al. Tim-3 enhances FcεRI-proximal signaling to modulate mast cell activation. *J Exp Med*. 2015 Dec;212(13):2289–304.
39. Harder KW, Parsons LM, Armes J, Evans N, Kountouri N, Clark R, et al. Gain- and loss-of-function Lyn mutant mice define a critical inhibitory role for Lyn in the myeloid lineage. *Immunity* [Internet]. 2001 Oct [cited 2018 Aug 3];15(4):603–15. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/11672542>
40. Fitzer-Attas CJ, Lowry M, Crowley MT, Finn AJ, Meng F, DeFranco AL, et al. Fcγ receptor-mediated phagocytosis in macrophages lacking the Src family tyrosine kinases Hck, Fgr, and Lyn. *J Exp Med* [Internet]. 2000 Feb 21 [cited 2018 Aug 3];191(4):669–82. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/10684859>
41. Ocaña-Guzman R, Torre-Bouscoulet L, Sada-Ovalle I. TIM-3 Regulates Distinct Functions in Macrophages. *Front Immunol* [Internet]. 2016 [cited 2018 Aug 3];7:229. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/27379093>
42. Jensen-Jarolim E, Bax HJ, Bianchini R, Capron M, Corrigan C, Castells M, et al. AllergoOncology - the impact of allergy in oncology: EAACI position paper. *Allergy* [Internet]. 2017 Jun [cited 2018 Jul

- 11];72(6):866–87. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/28032353>
43. Daniels TR, Leuchter RK, Quintero R, Helguera G, Rodríguez JA, Martínez-Maza O, et al. Targeting HER2/neu with a fully human IgE to harness the allergic reaction against cancer cells. *Cancer Immunol Immunother* [Internet]. 2012 Jul 30 [cited 2018 Jul 15];61(7):991–1003. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/22127364>
44. Fazekas-Singer J, Singer J, Ilieva KM, Matz M, Herrmann I, Spillner E, et al. AllergoOncology: Generating a canine anticancer IgE against the epidermal growth factor receptor. *J Allergy Clin Immunol* [Internet]. 2018 Sep [cited 2019 Mar 25];142(3):973–976.e11. Available from: <https://linkinghub.elsevier.com/retrieve/pii/S0091674918306924>
45. Plotkin JD, Elias MG, Fereydouni M, Daniels-Wells TR, Dellinger AL, Penichet ML, et al. Human Mast Cells From Adipose Tissue Target and Induce Apoptosis of Breast Cancer Cells. *Front Immunol* [Internet]. 2019 Feb 18 [cited 2019 Mar 25];10:138. Available from: <https://www.frontiersin.org/article/10.3389/fimmu.2019.00138/full>

Figure Legends

Figure 1. M1 and M2 macrophages are obtained through *ex vivo* differentiation of human monocytes

(a) Flowchart depicting the isolation and cell culture methodology to derive M1 and M2 macrophages *ex vivo*. (b) Flow cytometric analysis of M1 and M2-typical surface markers CD80, CD86, CD163, CD206 representing the percentage of positive cells to anti-human CD80-BV395, anti-human CD86-BV737, anti-human CD163-BV605, anti-human CD206-PE/Cy7 antibody respectively (average of 4 independent experiments \pm SEM). (c) Detection of Fc ϵ RI-II via flow cytometry on monocytes, M0, M1 and M2 *ex vivo* differentiated macrophages. Data represent the percentage of positive cells to anti-human Fc ϵ RI-PE, anti-human CD23-BV421 antibodies (average of 4 independent experiments \pm SEM). Statistical analysis obtained performing One-way ANOVA coupled with Tukey's post-test (** p -value<0.0005, ** p -value<0.005, * p -value<0.05).

Figure 2. Cross-linking of cell-bound IgE regulates macrophage secretion of soluble mediators

(a) Schematic representation of the cross-linking of IgE antibodies on macrophages. (b-e) Differentiated macrophages were incubated with SF-25 IgE or NIP IgE antibody alone for 30 minutes at 37°C or followed by a second 30 minutes incubation with anti-human IgE antibody in order to cross-link the cell-bound IgEs. (b) Quantification of Pro-inflammatory cytokines TNF α , IFN γ , IL-12, IL-1 β , (c) Anti-inflammatory cytokines IL-4, IL-6, IL-10, IL-13, (d) Interferon Gamma-induced chemokines MIG (CXCL9) and I-TAC (CXCL11) and (e) Macrophage chemoattractant chemokines MCP-1 (CCL2) and RANTES (CCL5) levels were analyzed via Luminex magnetic assay. Values are expressed as pg/mL (average of two independent experiments \pm SEM).

Figure 3. Kinase phosphorylation is differentially altered between M1 and M2 macrophages upon IgE cross-linking

(a) Images from Proteome Profiler Human Phospho-Kinase Array (decrease in phosphorylation marked in red; increase in phosphorylation marked in green). Each kinase is spotted in duplicate. (b) Schematic representation of the percentage of increase and decrease in phosphorylation levels in M1 and M2 macrophages upon IgE cross-linking. (c) Pixel densitometry analysis was expressed as fold change comparing the sample subjected to IgE cross-linking to the corresponding non-treated sample.

Figure 4. Human M1 and M2 macrophages display differential signaling cascade upon IgE engagement

(a) Fc ϵ RI pathway network generated via KEGG database. Highlighted in blue are kinases analysed via Human Phospho-Kinase Antibody Array. (b) List of implicated pathways upon IgE cross-linking, represented as the average fold change of implication between M1 and M2 macrophages. Data generated via Reactome.

Figure 5. Effects of IgE cross-linking on macrophage gene expression and correlation of LYN and TIM-3

expression of tumour patient prognosis

(a) Differentiated M0, M1 and M2 macrophages were treated with: SF-25 IgE alone, anti-human IgE or both. The effects of IgE cross-linking on gene expression of LYN and TIM-3 were detected by performing RT-PCR on mRNA content of treated and lysed cells. Values are expressed as fold change compared to the untreated cell sample of one single preliminary experiment. (b) Association between mRNA expression of crucial elements of FcεRI pathway LYN and TIM-3 and 5-year survival of patients in cohorts of gastric, lung and ovarian cancer were analysed. Graphs show Kaplan-Meier survival curves upon discrimination between high and low expression levels of genes of interest *Lyn* and *Tim-3*. Threshold was set to the upper quartile of patients' gene expression data: "high" expression represents the upper quartile, "low" expression encompasses the remaining data of the set.

Figure 6. IgE antibodies activate *ex vivo* monocyte-derived macrophages towards A-2058 melanoma cells

The tumour cell killing assay was performed in order to investigate a) the ability of IgE antibodies to activate *ex vivo* derived macrophages and b) whether the different phenotypes could reflect into different anti-tumour activity. (a) Representative flow cytometry plots depicting the gating strategy applied for detection of cytotoxic and phagocytic activity of macrophages. The black gate highlights the double positive cells, indicating phagocytic activity (CFSE+ tumour cells internalized by macrophages). The purple gate emphasizes the loss in the total number of tumour cells when incubated with SF-25 IgE antibody. (b) Tumour cell killing was investigated in PBS control conditions, with 5 µg/ml of SF-25 IgE, SF-25 IgG1 and negative (non-specific) (NIP IgE or IgG1) antibody controls. Values are represented as percentage of tumour killing (average of 2 independent experiments ± SEM; each condition was tested in triplicate). Statistical analysis obtained performing One-way ANOVA coupled with Bonferroni's post-test; ** p-value=0.0012, * p-value=0.01 (M1) and 0.03 (M2) (* p-value= 0.01) *=p-value<0.05, **=p-value<0.01

Figure 7. IgE antibody-mediated re-programing of human macrophages

Schematic picture illustrating the suggested model of repolarization of human macrophages via engagement and activation by IgE antibodies: quiescent M0 macrophages and anti-inflammatory M2 macrophages are skewed towards a new polarization phenotype, displaying both pro- and anti-inflammatory features.

Table 1. Summary of IgE antibody-mediated effects on human macrophage phenotypes

List of soluble mediators released, gene expression and kinase phosphorylation modifications engendered on human macrophage subsets upon IgE crosslinking. Quiescent M0 and M2 macrophages cross-linked with IgE antibodies acquire typical M1 marker features and are skewed towards a new polarization status (yellow macrophage). IgE cross-linked on the surface of M1 macrophages retains a pro-inflammatory, anti-tumour polarization status. The five most perturbed kinases amongst 43 analysed are reported in this table. The red arrow indicates increase/decrease of release, expression or phosphorylation level compared to baseline conditions.

Supplemental Material

Supplementary Figure 1 Gating strategy for monocyte purity evaluation

(a) Representative flow cytometry plots depict cell populations in sample fractions. (b) Level of monocyte purity in the isolated samples (n=4) via CD14 and CD16 staining. Values are represented as percentage of positive cells (average of n=3 independent experiments \pm SEM).

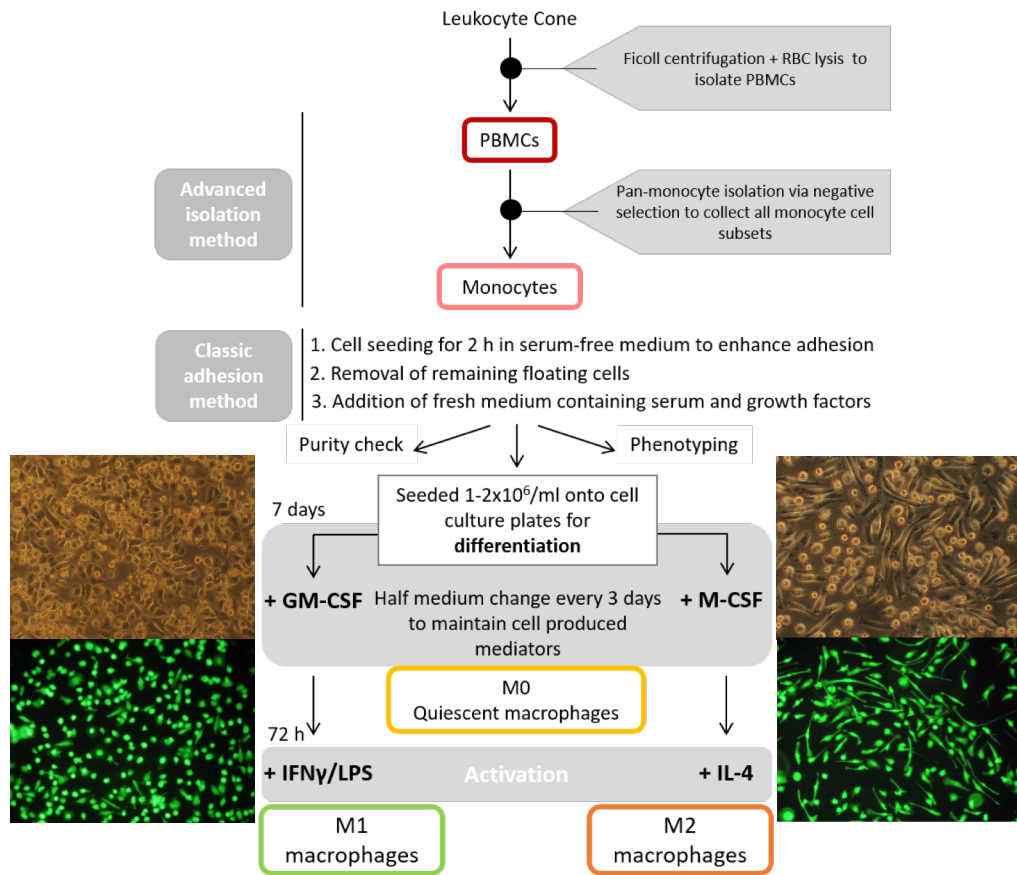
Supplementary Figure 2. Surface levels of endogenous IgE on differentiated macrophages

Representative flow cytometry plots of two independent experiments for quantification of endogenous IgE bound to differentiated macrophages, treated with anti-human IgE-FITC only or SF-25 IgE and anti-human IgE-FITC combined. FITC-positive cells are gated and relative percentage to whole cell sample is depicted.

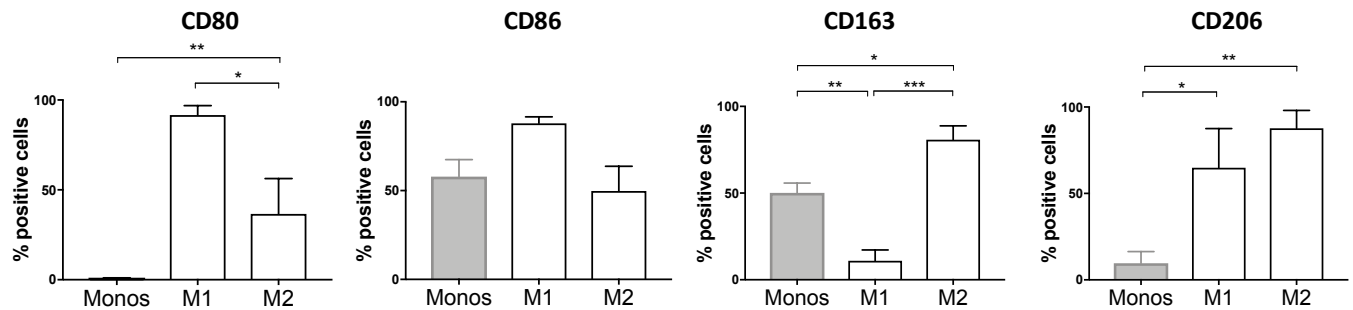
Supplementary Figure 3. Cross-linking of cell-bound IgE regulates surface marker levels of macrophages

Differentiated macrophages (n=3) were incubated with SF-25 IgE or NIP IgE antibody alone or in combination with anti-human IgE antibody. Monocyte, M1 and M2 typical surface marker levels were analyzed before and after treatment by staining the cells with fluorescently labelled antibodies targeting CD14, CD16, Fc ϵ RI, Fc ϵ RII (CD23), CD80, CD86, CD163 and CD206. Values are expressed as fold change compared to the non-treated sample \pm SEM, based on a number of 3 independent experiments.

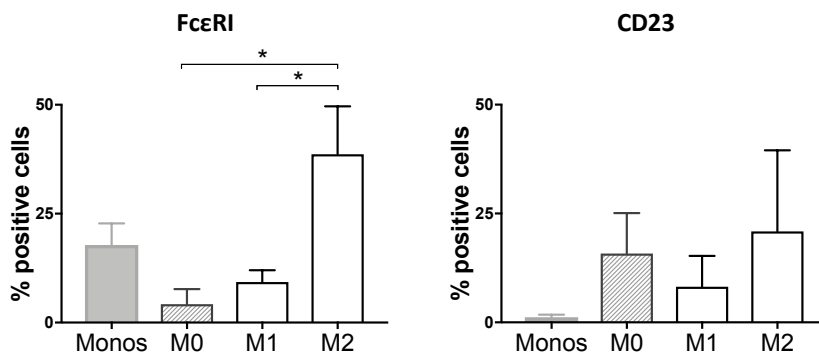
A

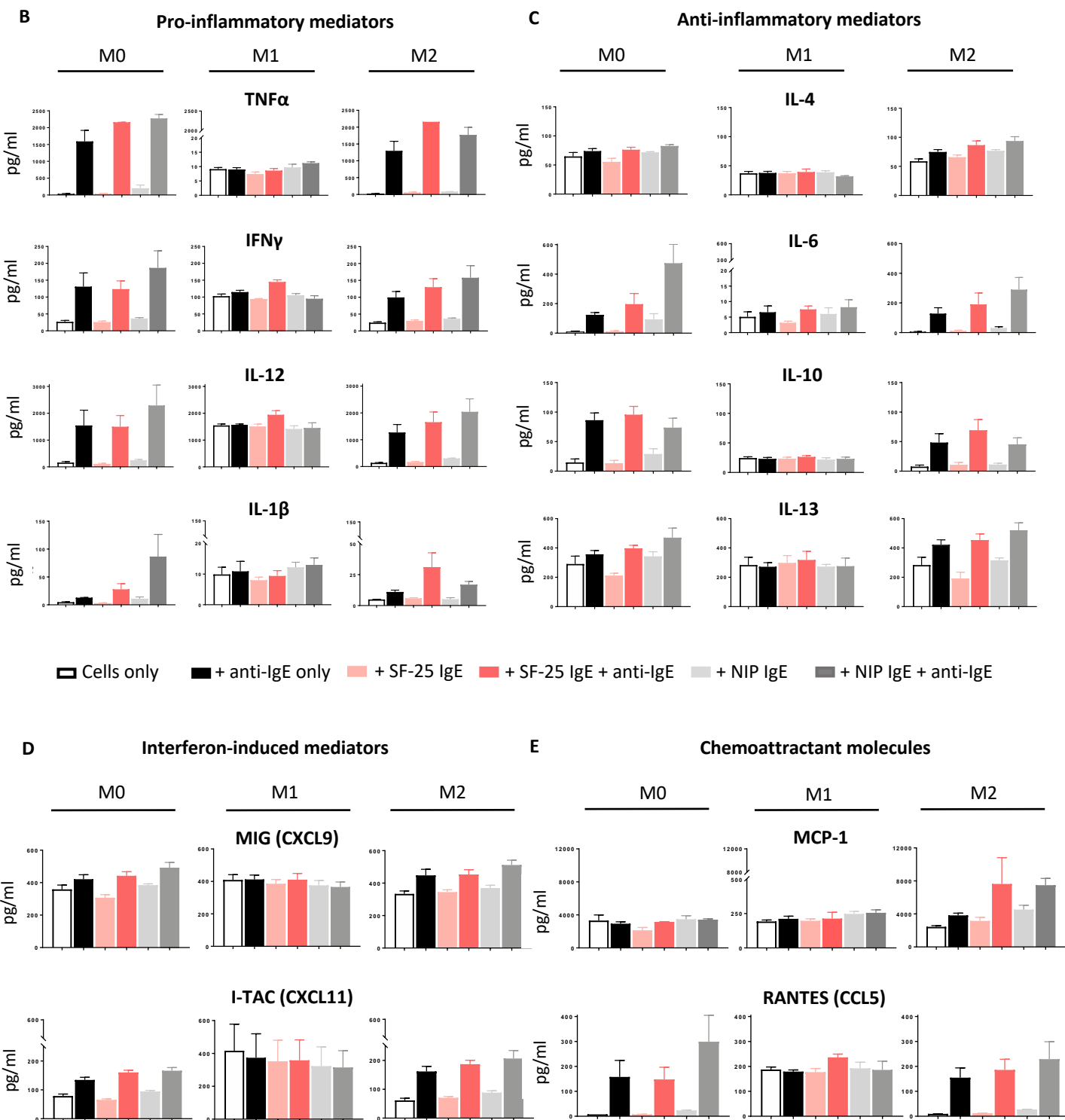
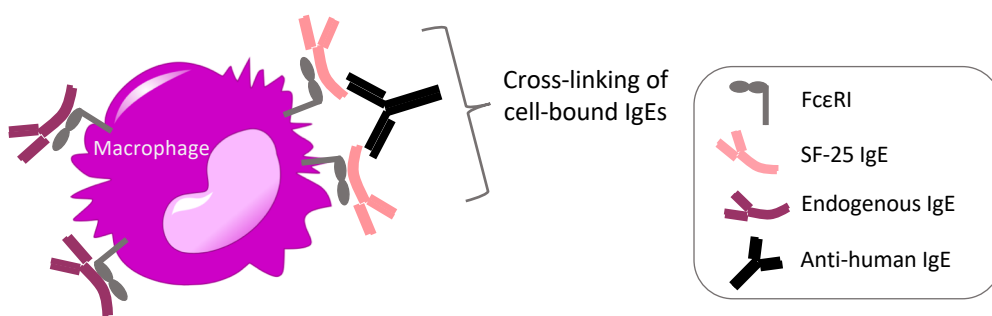


B

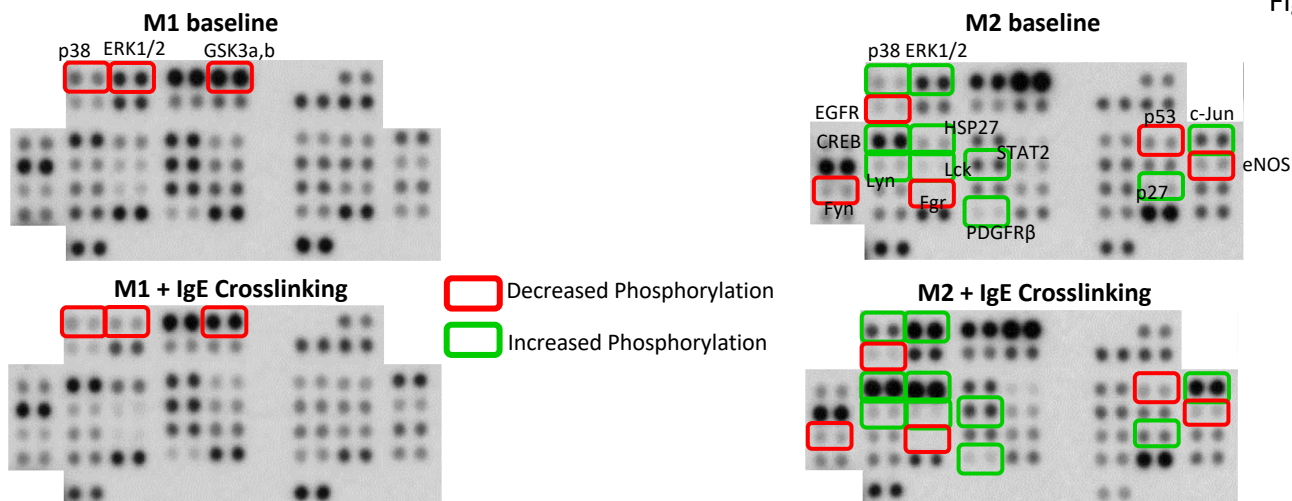


C

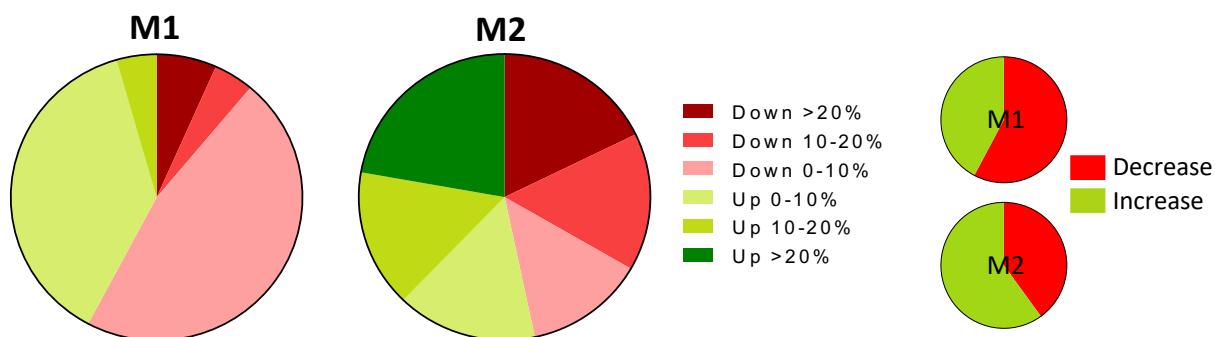




A



B



C

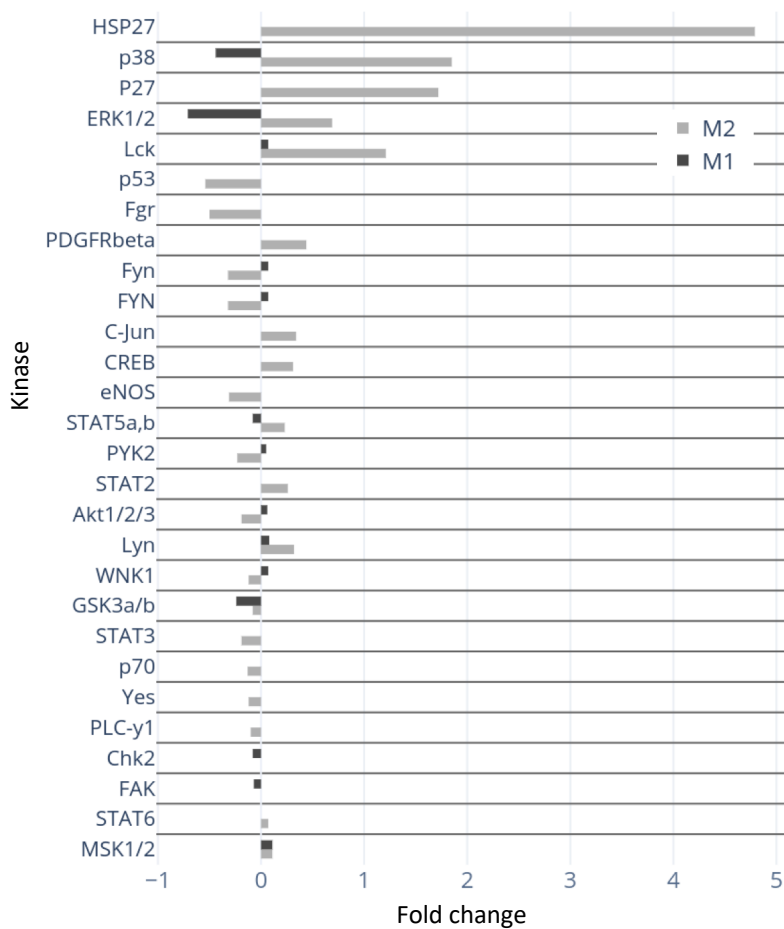
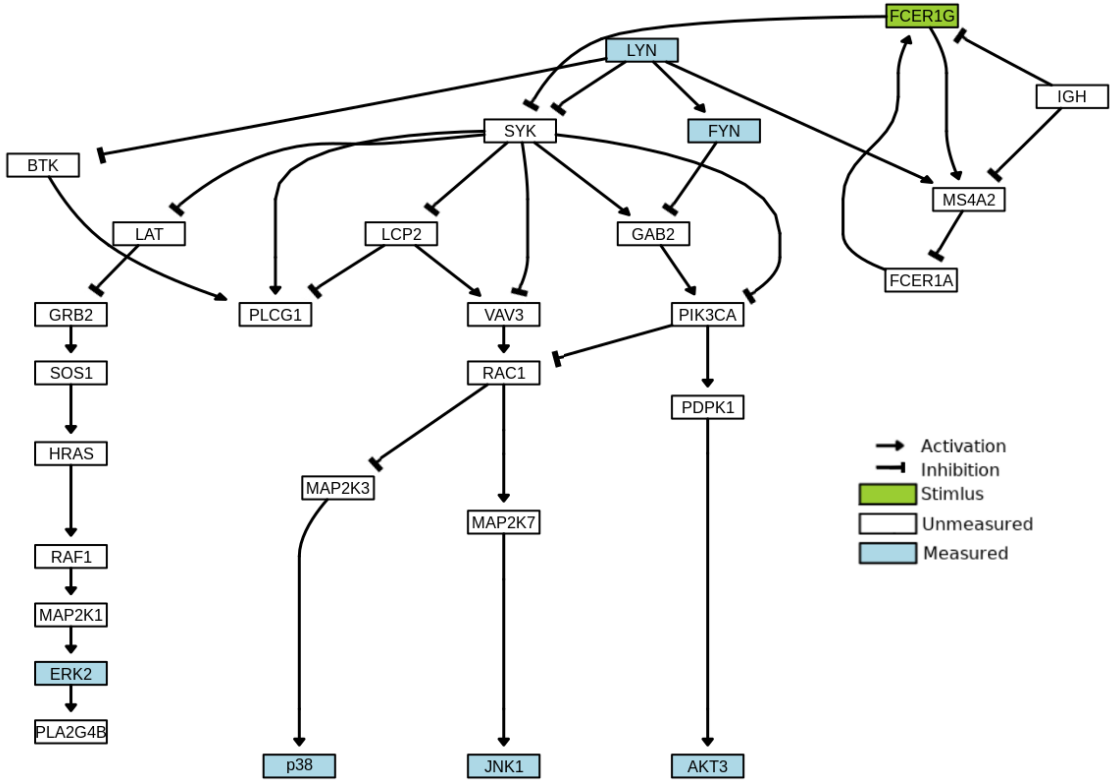
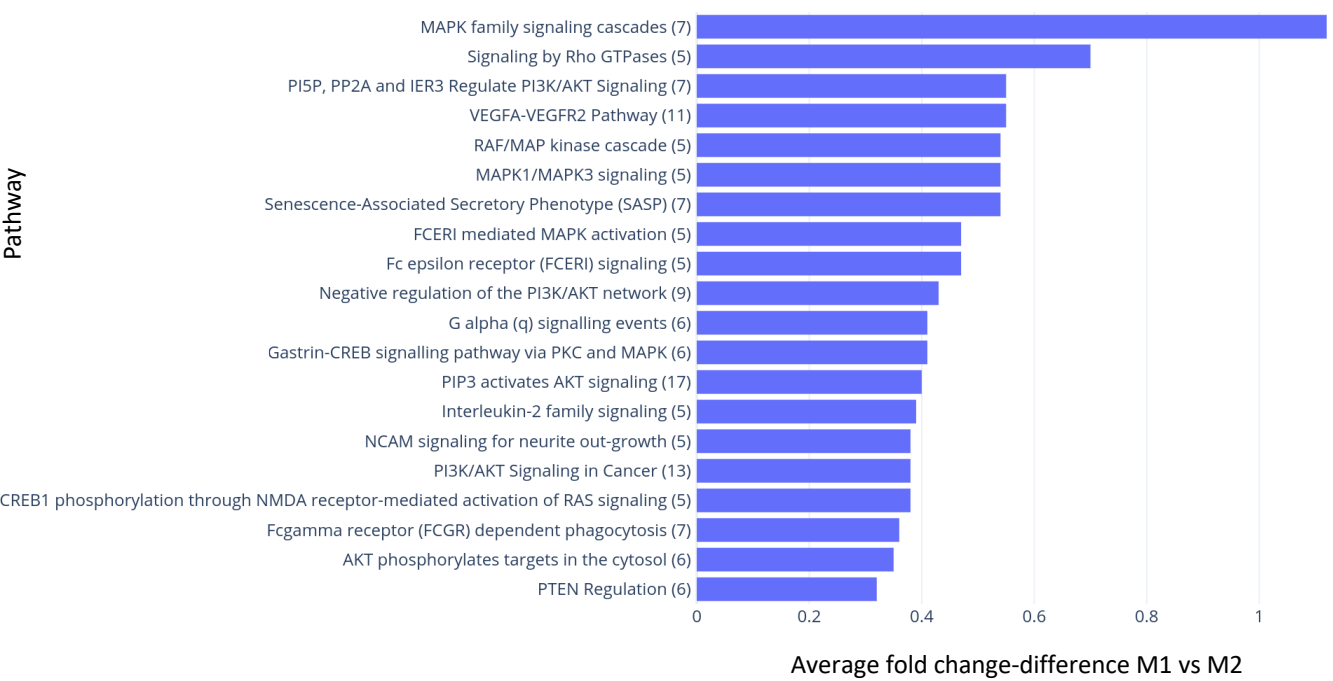


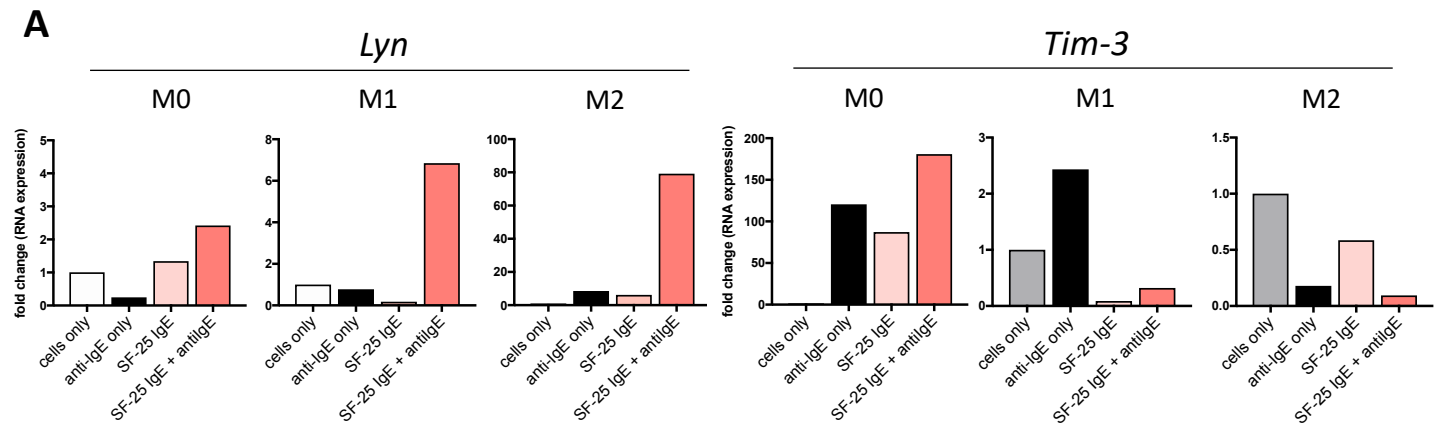
Fig 4

A



B





B

Solid tumour patient 5-year Overall Survival based on LYN and TIM-3 expression

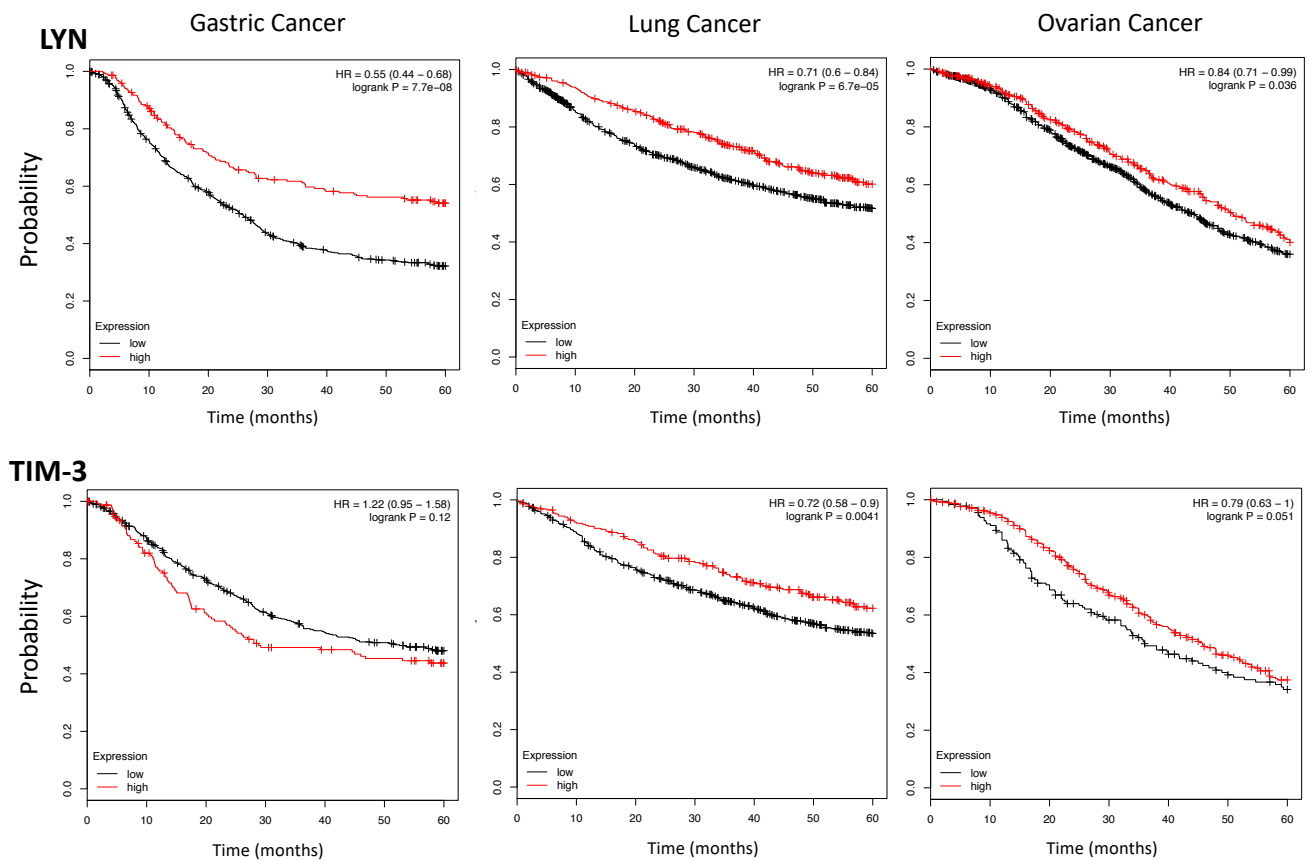
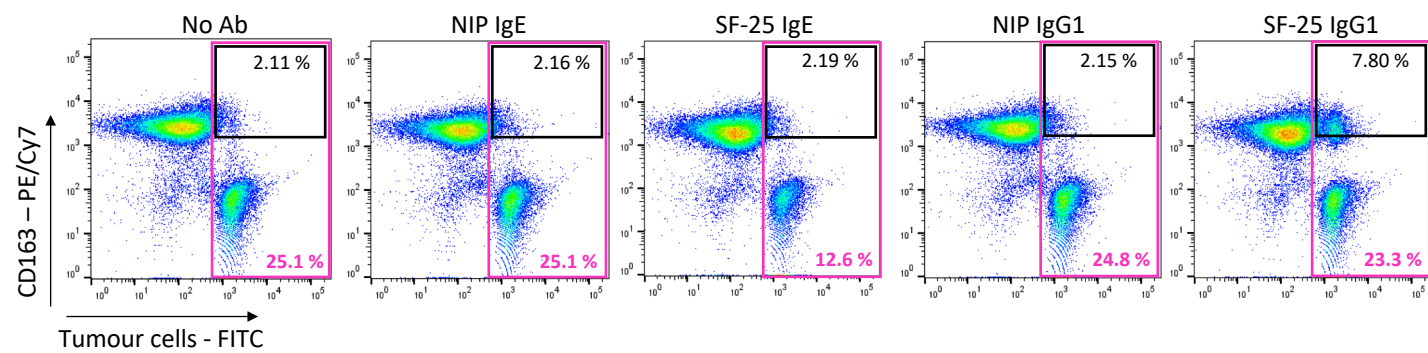
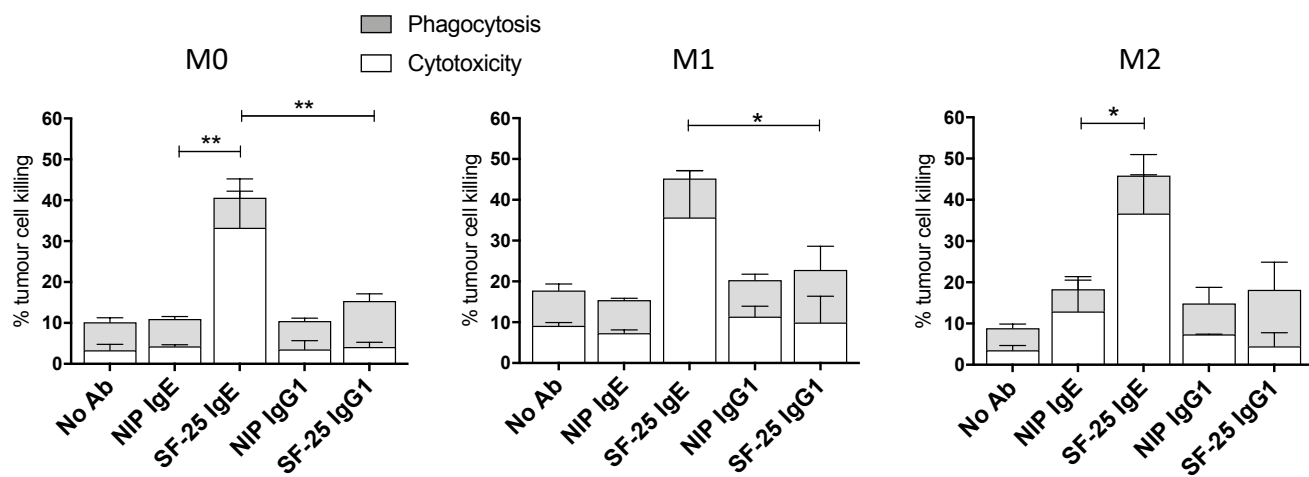
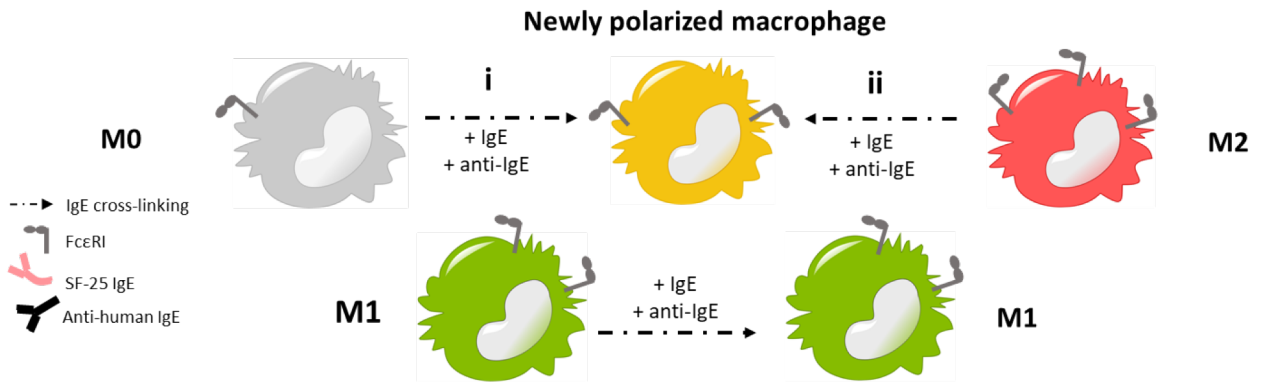


Fig 6

A**B**

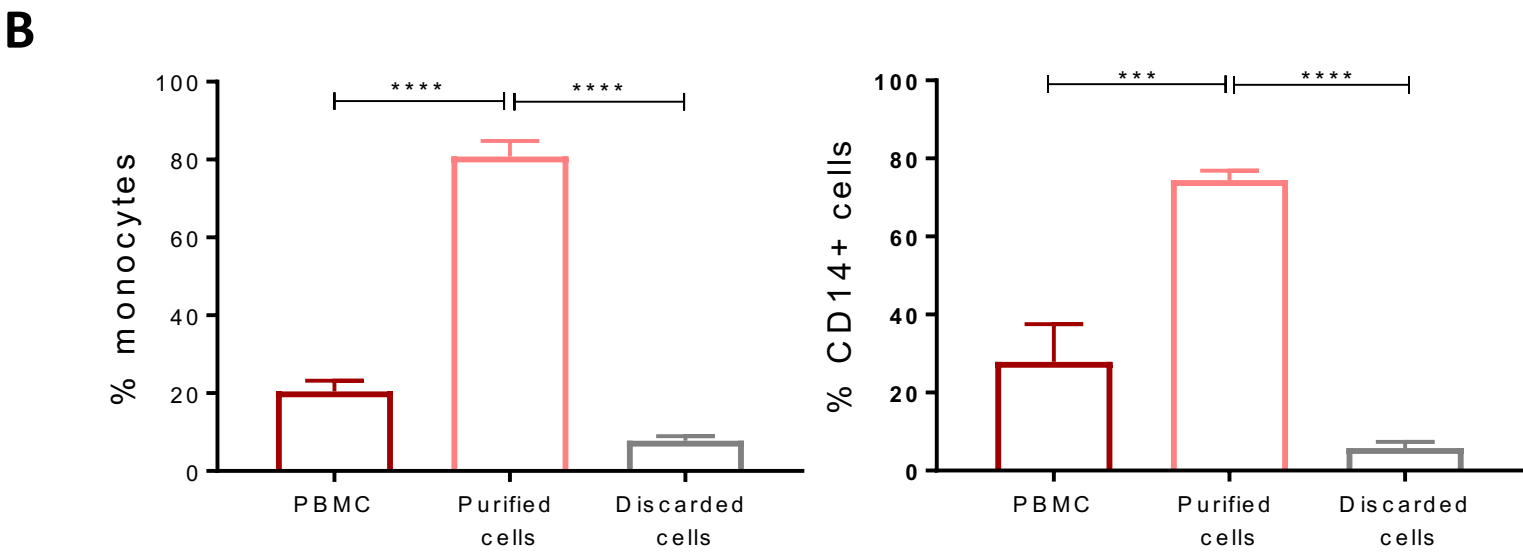
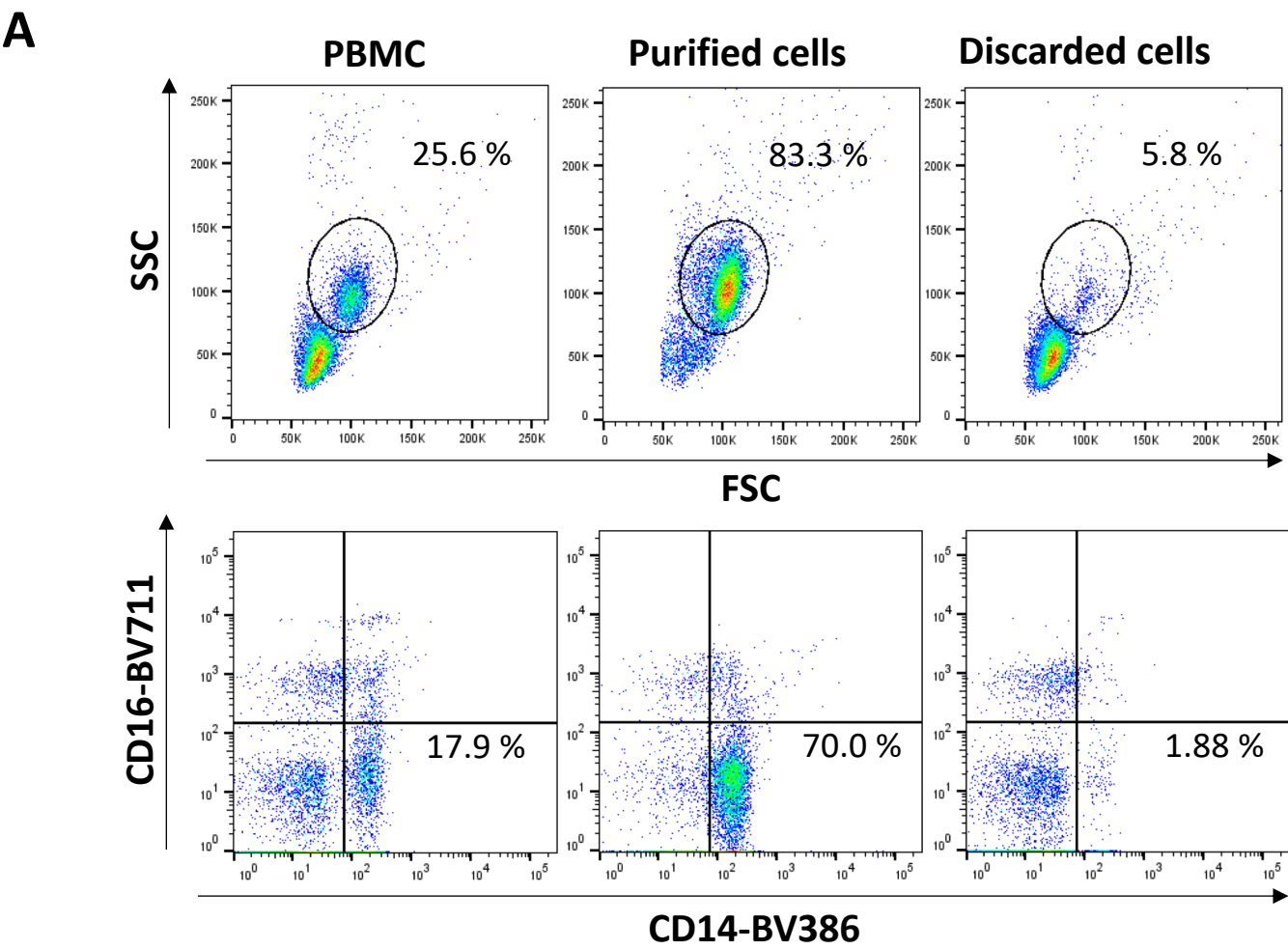


Summary of IgE antibody-mediated effects on human macrophage phenotypes

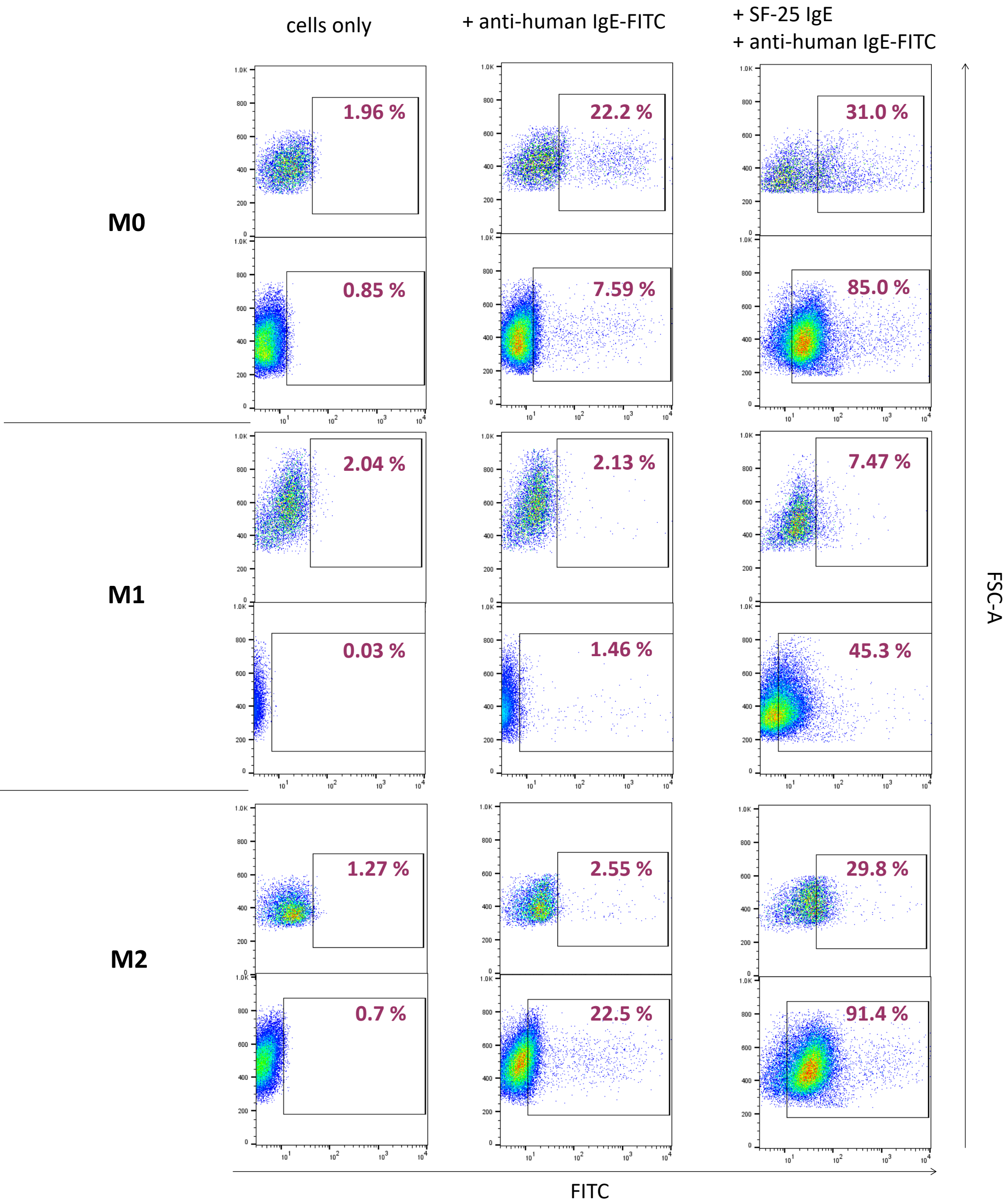
	Secreted Cytokines/Chemokines		Gene expression modulation upon IgE crosslinking	Phosphorylation upon IgE crosslinking	Final macrophage phenotype
	Physiological state / IgE engagement	IgE Cross-linking			
M0	TNF α IFN γ IL-1 β RANTES IL-10 IL-12 IL-13 CXCL9 CXCL11 MCP-1 IL-6 IL-4	↑ TNF α ↑ IFN γ ↑ IL-1 β ↑ RANTES ↑ IL-10 ↑ IL-12 ↑ IL-13 ↑ CXCL9 ↑ CXCL11 MCP-1 ↑ IL-6 IL-4	↑ Lyn ↑ Tim-3	N/T	Newly polarized macrophage phenotype
M1	TNF α IFN γ IL-1 β RANTES IL-10 IL-12 IL-13 CXCL9 CXCL11 MCP-1 IL-6 IL-4	TNF α IFN γ IL-1 β RANTES IL-10 IL-12 IL-13 CXCL9 CXCL11 MCP-1 IL-6 IL-4	↑ Lyn Tim-3	↓ ERK1/2 ↓ p38	M1
M2	TNF α IFN γ IL-1 β RANTES IL-10 IL-12 IL-13 CXCL9 CXCL11 MCP-1 IL-6 IL-4	↑ TNF α ↑ IFN γ ↑ IL-1 β ↑ RANTES ↑ IL-10 ↑ IL-12 ↑ IL-13 ↑ CXCL9 ↑ CXCL11 ↑ MCP-1 ↑ IL-6 IL-4	↑ Lyn ↓ Tim-3	↑ ERK1/2 ↑ p38 ↑ HSP27 ↑ p27 ↑ Lck	Newly polarized macrophage phenotype - The only phenotype with augmented MCP-1 secretion upon IgE cross-linking

Table 1. List of soluble mediators released, gene expression and kinase phosphorylation modifications engendered on human macrophage subsets upon IgE crosslinking. Quiescent M0 and M2 macrophages cross-linked with IgE antibodies acquire typical M1 marker features and are skewed towards a new polarization status (yellow macrophage). IgE cross-linking on M1 macrophages retains a pro-inflammatory, anti-tumour polarization status. The five most perturbed kinases amongst 43 analysed were reported in this table. The red arrow indicates increase/decrease of release, expression or phosphorylation level compared to baseline conditions.

Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3

